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# Reaktion des Äthylenoxyds auf Aminosäure und Eiweissstoffe

Von Kazuyuki MAEKAWA und Shinichi TSUMURA

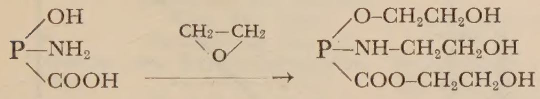
*Institut für biologische Chemie der landwirtschaftlichen Fakultät, Universität Ehime.*

Eingegangen am 9, Januar 1956

Ethylene oxide reacted for the first time with amino group of amino acid in alkali side under mild conditions, when magnesium ion was added. In the conditions N-hydroxyethylalanine was isolated from the reaction mixture consisting of alanine and ethylene oxide.

One mol of trypsin combined with 10 mols of ethylene oxide at 5°C, pH 8.0 in 12 days, but the change in the activity through combination was not plain.

Im Jahre 1944 hat Fraenkel-Conrat über die Reaktion des Epoxydes auf die Eiweissstoffe (Ovalbumin und β-Laktoglobulin) gesagt, dass Epoxyde auf alle funktionellen Gruppen des Eiweissstoffes in sauren sowie in alkalischen Bedingungen folgenderweise reagieren;



$$P\begin{cases}OH\\NH_2\\COOH\end{cases}\xrightarrow{CH_2-CH_2\ (O)}P\begin{cases}O-CH_2CH_2OH\\NH-CH_2CH_2OH\\COO-CH_2CH_2OH\end{cases}$$

Dabei erhielt er das Reaktionsprodukt als Niederschlag und stellte dessen Stickstoffgehalt und die Abnahme jeder funktionellen Gruppe[1] fest.

Aber diese Ergebnisse sind seither von Niemand nachgeprüft und nicht weiter entwickelt worden. Wir haben also diese Reaktion mit einem Fermente, welches bei alkalischem pH optimal wirken kann, probiert und dabei gehofft, die Blockierung desselben an Oxy-bzw. Aminogruppen zu beobachten. Merkwürdigerweise wurde jedoch fast kein Niederschlag erhalten und auch keine Abnahme an zugesetzter Epoxyde anerkannt.

Um den Epoxydering aufzuspalten, wobei 1,2-Glykol als ein produkt entsteht, braucht man meistens Wärme[2a], Säuren[2b], Alkali[2c], Metalloxyd[2d], usw.[2e]. Wir fanden, dass Magnesium- und Zinksalz als Katalysator von N-Oxyäthylierung in Hinsicht auf die Anwendbarkeit auf diese Arbeit mit Proteine und Enzyme das geeignetste Mittel ist, während Calcium- und Aluminiumsalz nicht tauglich. (Tabelle I)

Aus dem Versuche von Oxyäthylierung der Aminosäuren in Vorhandensein von Magnesiumsalz wurde dann der Schluss gezogen, dass die Aminosäuren sich bei pH 8.7 in ihren Aminogruppen leicht blockieren und in N-Alkoxylaminosäuren verwandeln lassen.

Auf Trypsin reagiert 10 Mol Äthylenoxyde und dadurch ging die proteolytische Aktivität des ersteren zum Teil verloren.

## BESCHREIBUNG DER VERSUCHE

**(1) Bestimmungsverfahren von Äthylenoxyde.** Im allgemeinen behandelt man Äthylenoxyd (ÄO) mit Säuren, welche einen gewissen Katalysator wie $MgCl_2$ oder $CaCl_2$ enthält, und titriert das dabei entstandene Chlorhydrin mit Alkali[3]. Da aber diese Methode zur Bestimmung kleiner Menge ÄO ungeeignet war, machten wir folgendermassen vom Verfahren mit Perjodat[4] und Chromotropsäure

---

[1] H. Fraenkel-Conrat; *J. Biol. Chem.*, **154**, 227 (1944).

[2a] I.K.D. Petrov und E.S. Lagucheva; *Zhur. Obshchei Khim.*, **21**, 1247 (1951); *C.A.* **46**, 1959 (1952). T. Ishiguro, S. Kato und Y. Akazawa; *J. Pharm. Soc. Jap.*, **63**, 282 (1943).

[2b] A.M. Eastham, E.L. Blackall und G.A. Latremouille; *J. Am. Chem. Soc.*, **77**, 2182 (1955).

[2c] S.A. Miller, B. Bann und R.D. Thrower; *J. Chem. Soc.* **1950**, 3628. M. De Groote; U.S. Pat., 2, 652, 394. R. Oda; *Mem. Fac, Eng. Kyoto Univ.*, **14**, 195 (1952). A.E. Broderick; U.S. Pat., 2,682,535.

[2d] Y.K. Yur'ev, K.Y. Novitskii und E.I. Mingulina; *Doklady Akad, Nauk., SSSR*, **74**, 87 (1950); *C,A*, **45**, 3846 (1951).

M.S. Malinovskii und S.N. Baranov; *Zhur. Priklad. Khim.*, **25**, 410 (1952); *C.A.*, **47**, 2694 (1953).

M.S. Malinovskii et al.; *C.A.*, **48**, 11415; 8785 (1954).

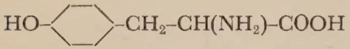
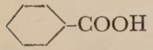
[2e] M.J. Kelley und J. Levy; U.S. Pat., 2, 681, 354.

[3] J.L. Jungnickel, E.D. Peters. A. Polgár und F.T. Weiss; Organic Analysis, Interscience Publisher, New York, Vol. I.S. 129 (1953).

[4] A.M. Eastham und G.A. Latremouille; *Can. J. Research*. B. **28**, 264 (1950).

TABELLE I

REAKTION DES ÄO MIT ORGANISCHEN SÄUREN UND ALKOHOLE

| Substanz | Ange-wandt (mg) | Lösungs mittel (ml) | pH | Zuge-setztes ÄO (mg) | Nach 3 Tagen bei 15°C | |
|---|---|---|---|---|---|---|
| | | | | | Rückbleibendes ÄO (mg) | Abnahme der funktionellen Gruppen (%) |
| $CH_3\cdot CH(NH_2)\text{-}COOH$ | 89 | Wasser (5) | 8.6 | 150 | 150 | 0 |
|  HO-⟨ ⟩-$CH_2\text{-}CH(NH_2)\text{-}COOH$ | 181 | 〃 | 8.6 | 150 | 150 | 0 |
| $CH_3\cdot CH_2\text{-}CH_2OH$ | 120 | 〃 | 8.6 | 300 | 300 | 0 |
| $H_2N$-⟨ ⟩-$SO_3H$ | 191 | 〃 | 8.6 | 150 | 144 | 4 |
| $H_2O$ | | 〃 | 5.5 | 150 | 150 | |
|  ⟨ ⟩-COOH | 112 | 50% Aceton (5) | | 150 | 10 Tage lang bei 20°C | 2.8 |
| $HOOC\text{-}(CH_2)_4\text{-}COOH$ | 146 | | | 150 | | 5.4 |

Gebrauch. Zuerst wurde nämlich die Probelösung mit verd. Schwefelsäure versetzt und das entstandene Äthylenglykol wurde durch Perjodat in Formaldehyd übergeführt, welches durch Erhitzen mit Chromotropsäure und Schwefelsäure eine Farbentwickelung erzeugt und sich kolorimetrisch bestimmen lässt.

Dem 10 ml vorher gewogenen und abgekühlten Wasser wurde in einem Röhrchen etwa 1 ml auf 0°C abgekühltes ÄO gegeben. Es wurde sofort völlig geschüttert, dann zugeschmolzen und aufs neue abgewogen. Anderseits wurde eine gewisse Menge (5–10 ml) der Probelösung bzw. des Wassers (Leerversuch) in ein mit Schliffstopfen versehenes Röhrchen gebracht. Dazu wurde die obige wässrige Lösung der ÄO mit abgekühlter Pipette hinzugesetzt. Je 1 ml aliquoter Teil wurde zuweilen dem Reaktionsgemisch entnommen und mit 5 ml 0.2 N Schwefelsäure versetzt. (die Abszisse der Figur 1 zeigt mg ÄO in ml der Lösung.). Nach halbstündigem Stehenlassen wurde 1 ml davon in einen Zentrifugebecher gebracht und mit 1 ml 10% iger $NaJO_4$ Lösung versetzt. Nach weiterem 1 stündigem Stehenlassen wurde das druch $NaJO_4$-Oxydation entstandene Formaldehyd in gewöhnlicher Weise bestimmt.

Hierzu wurden nämlich 2 ml 15%-iger Bleiacetatlösung und 5 ml Wasser hinzugefügt, um Sulfat, Jodat und Perjodat niederzuschlagen[5]. Aus überstehender Flüssigkeit nach Zentrifugieren wurde 1 ml aliquoter Teil pippetiert und dazu wurden 0.5 ml 3%-iger Chromotropsäure und 6 ml von konz. Schwefelsäure hinzugesetzt, und 30 Minuten lang im kochenden Wasserbad stehen gelassen. Man füllte es dann nach Abkühlen auf 25–100 ml mit Wasser.

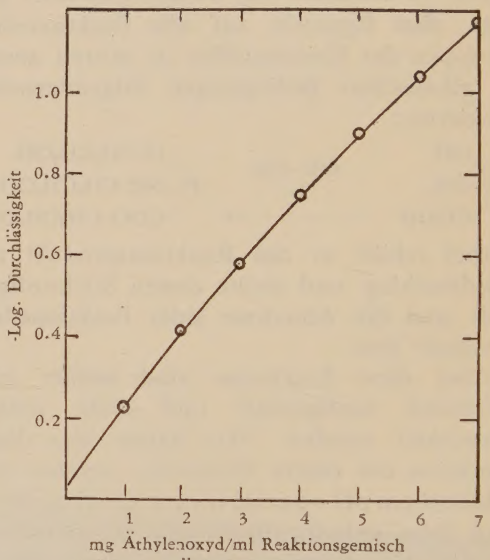


FIG. 1. Äthylenoxyd-Eichkurve.

(Mittelwerte von vier unabhängig von einander durchgeführten Bestimmungsserien.)

Schichtdicke 1 cm, Wellenlänge 570 mμ (Filter)

Die Extinktion der Lösung wurde in einer Küvette mit 1 cm Schichtdicke bei der Wellenlänge 570 mμ gemessen. Figur 1 gibt die Beziehungen zwischen der Extinktion der Reaktionslösung und der Konzentration des ÄO. Aus der Differenz zwischen blindem Versuche und der Probelösung liess sich die Menge des mit Probe verbundenen ÄO berechnen.

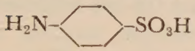
**(2) Reaktion des ÄO auf Aminosäuren, Alkohol, organische Säuren unter milden Bedingungen.** Die letzteren Substanzen wurden bei 0–20°C, pH 4.0–

---

[5] W.A. Vail und C.E. Bricker; *Anal. Chem.*, **24**, 977 (1952).

TABELLE II

EFFEKT DER KATALYSATOREN

| Substanz | Bedingungen | Abnahme der $NH_2$- bzw. HO-Gruppe | | | |
|---|---|---|---|---|---|
| | | Zn-Salz | Al-Salz | Mg-Salz | Ca-Salz |
| $CH_3 \cdot CH(NH_2)\text{-}COOH$ | pH 8.6, 20°C., nach 3 Tagen | 30% | 2% | 42% | 10% |
| $CH_3 \cdot CH_2 \cdot CH_2OH$ | 〃 | 10 | 0 | 25 | 3 |
|  | 〃 | 28 | 0 | 40 | 5 |

Jades Salz wurde zum Reaktionsgemische je 50 mg hinzugefügt.

TABELLE III

REAKTION DES ÄO MIT ALANINE IN GEGENWART VON $Mg^{\cdot\cdot}$

| Versuchs Nr. | Angew. Alanin Lösung* | Zugesetzte ÄO-Lösung** | $Mg^{\cdot\cdot}$-Zusetzen*** | Wasser | pH von Reaktions-gemischen |
|---|---|---|---|---|---|
| 1 | 5 | 5 | 1 | — | 8.5 |
| 2 | 5 | 5 | — | 1 | 5.0 |
| 3 | 5 | — | — | 6 | 5.0 |
| 4 | 5 | — | 1 | 5 | 8.5 |
| 5 | — | 5 | 1 | 5 | 8.5 |
| 6 | — | 5 | — | 6 | 5.0 |

| Versuchs Nr. | Extinktion bei Messung von ÄO, unter Anwendung von 1 ml des Reaktionsgemisches | | Nach 6 Tagen bei 10° | | | |
|---|---|---|---|---|---|---|
| | Nach 2 Tagen bei 10° | Nach 5 Tagen bei 10° | Verbundenes ÄO Mol / Mol Alanin | Rückbleibende $NH_2$-Gruppe# | Rückbleibender Formol-N | Rückbleibende Carboxyl-gruppe |
| 1 | 0.699 | 0.495 | 0.58 | 48% | 47.2% | 100% |
| 2 | 0.846 | 0.838 | 0.02 | 95.2 | 94.5 | 100 |
| 3 | 0.076 | 0.076 | 0 | 100 | 100 | 100 |
| 4 | 0.076 | 0.076 | | | | |
| 5 | 0.810 | 0.770 | | | | |
| 6 | 0.854 | 0.854 | | | | |

* Die Probelösung enthält 89 mg Alanin/ml.

** Die Lösung enthält 61 mg ÄO/ml.

*** Die Lösung enthält 83 mg $Mg^{\cdot\cdot}$/ml und wird zu pH 8.5 eingestellt.

# Es wurde mittels Ninhydrins bestimmt.

8.7 zusammen mit der verdünnten wässrigen ÄO-Lösung stehen gelassen. Das Ergebnis war jedoch, wie aus der Tabelle I ersichtlich ist, vollkommen negativ und die merkliche Reaktion wurde an keinen Substanzen beobachtet. Nach 10 Tagen reagierte höchstens nur 3–5% von der Carboxylgruppe der Säuren.

**(3) Effekt des Katalysators auf Reaktion des ÄO.** Mit der Absicht, die Reaktion des ÄO zu aktivieren, setzten wir zu diesem Systeme bei pH 8–8.5 eine ganz kleine Menge des Salzes der leichten Metalle bzw. Alkalierden zu. Wir bestimmten auch die Abnahme der funktionellen Gruppen, weil es sich leicht überlegen lässt, dass ÄO an und für sich durch Metallsalz polymerisieren und wegen Absorption scheinbar abnehmen kann. Tabelle II gibt ein Ergebnis, dass das Mg-Salz besonders eine grosse Wirkung hat.

**(4) Reaktion des ÄO auf Aminosäuren in Anwesenheit von Magnesiumsalz.** (a) Unter den so bestimmten Bedingungen behandelten wir zuerst Alanin mit ÄO. Das dabei gewonnene Ergebnis ist in der Tabelle III zusammengefasst. Von dem Reaktionsgemische wurden das zurückbleibende ÄO, die noch nicht reagierte $NH_2$-Gruppe, der Formol-Stickstoff und die Carboxylgruppe jedes für sich bestimmt. Die

TABELLE IV

REAKTION DES TRYPSINS MIT ÄO

| Versuchs Nr. | Trypsin Lösung* | Zugesetzte ÄO-Lösung** | Zugesetztes Mg$^{\cdot\cdot}$*** | Puffer Lösung (pH 8.0) | pH von Reaktionsgemische |
|---|---|---|---|---|---|
| 4 a | 8 | 5 | 1 | — | 8.0 |
| 4 b | 8 | 5 | — | 1 | 〃 |
| 4 c | 8 | — | 1 | 5 | 〃 |
|  | — | 5 | 1 | 8 | 〃 |
|  | — | 5 | — | 9 | 〃 |

| Versuchs Nr. | Reaktions dauer (bei 5°) | Verbundenes ÄO Mol / Mol Trypsin | Rückbleibende |  | Absorptionsspektren |  |  |  | Proteolytische Aktivität### |
|---|---|---|---|---|---|---|---|---|---|
|  |  |  | $NH_2$-Gruppe# | Tyrosyl u. Imidazol-Gruppe## | $\lambda_{max}$ m$\mu$ | $\varepsilon_{max}$ $\times 10^{-3}$ | $\lambda_{min}$ m$\mu$ | $\varepsilon_{min}$ $\times 10^{-3}$ |  |
| 4 a | 12 Stdn. | — | 83.4% | 100% | 275 | 62.4 | 250 | 22.6 | 100% |
| 4 b |  | — | 99.5 | 〃 | 〃 | 〃 | 〃 | 〃 | 100 |
| 4 a | 4 Tage | 7.2 | 24.5 | 〃 | 〃 | 〃 | 〃 | 〃 | 68.4 |
| 4 b |  | — | 96.0 | 〃 | 〃 | 〃 | 〃 | 〃 | 49.3 |
| 4 a | 12 Tage | 10.8 | 8.6 | 〃 | 〃 | 〃 | 〃 | 20.1 | 50.5 |
| 4 b |  | 1.4 | 94.7 | 〃 | 〃 | 〃 | 〃 | 22.6 | 41.2 |

* 200mg von kristallisiertem Trypsin (Mochida Akt. Ges.) wurde mit 25ml 0.05 M. Phosphat Puffer von pH 8.0 gelöst.

** Die Lösung enthält 29.6mg AO/ml.

*** Die Lösung enthält zirka 50mg Mg$^{\cdot\cdot}$/ml und wurde zu pH 8.0 neutralisiert.

# Es wurde mittels Ninhydrins bestimmt.

## Es wurde danks Folinsches Phenol-reagens bestimmt.

### Die Aktivität wurde nach Hämoglobin Methode7) bestimmt.

Aminogruppe reagierte beinahe 50% bei 10°C nach 6 Tagen, während die Carboxylgruppe fast ganz zurückblieb.

(b) Papierchromatographie: Nachdem Leucin und Alanin mit ÄO nach obiger Weise behandelt worden waren, wurden die Aminosäurederivate aus Reaktionsgemische papierchromatographisch festgestellt. Zur chromatographischen Trennung gebrauchten wir als Lösungsmittel wassergesättigtes *n*-Butanol und Essigsäure (4–1, Vol.-Tle.). Die Lokalisation der Flecken wurde zuerst durch Bespülen der Perjodatlösung, dann ammoniakalischer Silbernitratlösung, oder Nesslersches Reagens durchgeführt. Im weiteren Falle wurden $CS_2$ und Alkali, dann Na-Nitroprussid zum Elut des ausgeschnittenen Fleckes hinzugefügt. Die $R_F$-Werte der Verbindungen waren wie folgende: N-Oxyäthylleucin; 0.28, N-Oxyäthylalanin; 0.42, Äthylenglykol; 0.94.

(c) Isolierung von N-Oxyäthylalanin aus dem Reaktionsgemische: Alanin (1.8 g) wurde in 10 ml Wasser gelöst, mit NaOH zu pH 8.7 reguliert und mit 1 ml Mg-Salzlösung von demselben pH (etwa 100 mg Mg enthalten) und weiter mit 5 ml 2 g ÄO enthaltendem Wasser versetzt. Nachdem das Gemisch 15 Tage lang aufbewahrt worden war, wurde das überschüssige ÄO durch Lüftung entfernt. Dann wurde mit Salzsäure angesäuert und 5 ml von 40%-iger $NaNO_2$-Lösung hinzugefügt, um Aminogruppe in Oxygruppe zu verwandeln. Durch diese Behandlungen ging die sekundäre Amine in die Nitroso-Verbindung über.

Nachdem Milchsäure durch Ausziehung mit Äther entfernt worden war, wurde die Lösung mit 10 ml von 2 N $H_2SO_4$ auf dem Wasserbade 1 Stunde lang erhitzt, um Nitrosogruppe zu beseitigen. Hierbei liess sich aber papierchromatographisch nachweisen, dass Alanin zum Teil wiederkehrt. Dann wurde dies mit $Ba(OH)_2$ zu pH 4.5 neutralisiert und abgedampft. Der Rückstand wurde mit *n*-Propanol extrahiert. Die weitere Trennung fand durch Papierchromatographie (BuOH gesätt. mit $H_2O$ : AcOH=4 : 1, $R_F$ 0.42) statt und die einzelnen Substanzen wurden aus zerschnittenem Papiere mit Wasser extrahiert. Aus dem Elute von $R_F$ 0.42 kristallisierte sich durch Verdampfung allmählich eine Verbindung von F. 193°C.

$C_5H_{11}O_3N$ Ber.: C, 45.11; H, 8.27%

Gef.: C, 45.89; H, 8.08%

Dies stimmte mit dem von Kiprianov6) berichteten

---

6) A.I. Kiprianov und B.A. Rashkovan ; *J. Gen. Chem.,(USSR)*, **7**, 1026 (1937); *C.A.*, **31**, 5356 (1937).

7) M.L. Anson; *J. Gen. Physiol.*, **22**, 79 (1938).

N-Oxyäthylalanin überein. Es wurde unter Vakuumdestillation bei 116°/5 mm überdestilliert, und dabei entstand wahrscheinlich 3-Methyl-2-Morpholinone. Beim Anzünden riechte es nach Pyridin.

Pikrat wurde in gewöhnlicher Weise dargestellt. Nach Umkristallisation aus Wasser erhielt man feine, gelbe Nadeln, F. 152°C.

(5) **Reaktion des ÄO auf Trypsin.** In Anwesenheit von wenigem Mg-Salz wurde Trypsin mit ÄO unter den in der Tabelle IV gegebenen Bedingungen behandelt. Das verbrauchte ÄO, die rückbleibende Aminogruppe und die Abnahme der enzymatischen Aktivität wurden einzeln bestimmt. Beinahe 10 Mol von ÄO verband sich nämlich mit pro Mol Trypsin und die Aminogruppen nahmen zu 8% ab, aber Tyrosylgruppe blieb unverändert zurück. Die Aktivität ging beinahe 50% verloren. Da die Aktivität in der Probe des Versuchs 4 b (In der Tabelle IV) auch verloren ging, scheint es zweifelhaft, ob die Abnahme der Aktivität auf die Vereinigung mit ÄO zurückzuführen sei. UV-Absorptionsspektrum zeigt durch Behandlung mit ÄO keine Änderung.

Trotz wiederholter Versuche ist es jedoch nicht gelungen, modifiziertes Trypsin zu kristallisieren.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 3, p. 106~109, 1956]

# Einwirkung von Äthylenoxyden auf Lysozym

Von Kazuyuki Maekawa und Masahisa Kushibe
*Institut für biologische Chemie der Landwirtschaftlichen Fakultät, Universität Ehime*

Eingegangen am 9, Januar 1956

One mol. of lysozyme combined with 12 mols. of ethylene oxide at 4–5°C, pH 8.7, when magnesium ion existed.

Lysozyme, after having reacted with ethylene oxide so thoroughly that amino groups were no more detectable, still did not alter in the lytic activity and UV-absorption. Only in the ionophoresis a different result appeared. This N-hydroxyethyl derivative of lysozyme behaved also almost equally with the native lysozyme in the oxidation by periodate.

Schroeder berichtete, dass die Aktivität vom Lysozyme fast zurückbleibt, als man dessen Aminogruppe mit 2,4-Dinitrofluorobenzol blockiert1). D. De Fontaine et al. liessen Phenylisothiocyanat auf Lysozym reagieren, um dessen Amino-Ende zu bestimmen, aber sie beschrieben nichts weiter in Bezug auf die Veränderung seiner Aktivität[2]. Neuerdings behandelten Dickman et al. das Lysozym mit Xanthydrol und bekamen Xanthyllysozyme, welches im geraden Verhältnisse zu der Mange zugesetztes Xanthydrols seine Aktivität verliert, und weiter durch Hydrolyse desselben Monoxanthyltryptophan[3].

Hinsichtlich der Reaktion des Äthylenoxyds auf Lysozym gibt es jedoch neuerdings keinen Versuch. Wir beschäftigten uns also mit der Reaktion von Lysozyme mit Äthylenoxyde nach der Weise, wie in vorläufiger Mitteilung vorgestellt ist, um seine Aminogruppe zu blockieren.

Das so zur Genüge verbundene Lysozyme, wie keine freien Aminogruppen mehr erkennbar sind, hat auch gänzliche Aktivität und zeigt im Vergleich mit nativem Lysozyme ganz ähnliches Absorptions-spektrum. Es verhält sich aber verschiedentlich bei der Papierionophorese; das behandelte Lysozym wandert nämlich bei pH 4.0 nicht, während Lysozyme sich nach Kathode fortbewegt. (Fig. 1)

In dem Versuche von Perjodat-Oxydation des mit Äthylenoxyde blockierten Lysozyme kamen wir zum gleichen Resultate mit den schon von uns berichteten Ergebnissen über das Lysozym4).

Aus diesen Tatsachen lässt sich folgern, dass sowohl die am Ende stehenden Aminogruppe, einschliesslich ε-Aminogruppe, als auch die Oxyaminosäure mit lytischer Aktivität keinesfalls zu tun haben.

FIG. 1. Elektrophoregramm vom ÄO-Lysozyme.
a) Kontrol (natives Lysozym)
b) ÄO-Lysozym
Michaelis'cher N/10 Tartrate-Puffer (pH 4.0), 500 Volt, 4.8–9.5 mA/4 cm, 3 Stdn. $HgCl_2$-BPB-Färbung.

## BESCHREIBUNG DER VERSUCHE

### (a) Reaktion des Äthylenoxyds auf Lysozym.

Mehrfach umkristallisiertes Lysozym wurde in verdünnter Essigsäure von pH 4.5 derart aufgelöst, dass die Konz. der Enzymlösung bei 5–7 mg/ml blieb

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1) W. A. Schroeder; *J. Am, Chem. Soc.*, **74**, 5118 (1952).

2) D. De Fontaine und S. W. Fox; ebenda, **76**, 3701 (1954).

3) S. R. Dickman, R. B. Kropf und C. M. Proctor; *J. Biol. Chem.*, **210**, 491 (1954).

4) K. Maekawa und M. Kushibe; *Bull. Agric. Chem. Soc. Japan*, **19**, 28 (1955).

TABELLE I

REAKTION DES LYSOZYMS MIT ÄTHYLENOXYDE

(Ansatz der Versuche)

| Versuchs Nr. | Lysozym Lösung* | Zugesetzte ÄO Lösung** | Zugesetztes $Mg^{\cdot\cdot}$ | Puffer Lösung (pH 8.7) |
|---|---|---|---|---|
| 1 | 10 | 5 | 1 | — |
| 2 | 10 | 5 | — | 1 |
| 3 | 10 | — | 1 | 5 |
| 4 | — | 5 | 1 | 10 |
| 5 | — | 5 | — | 11 |

| Versuchs Nr. | Reaktionsdauer | Verbundenes ÄO Mol / Mol Lysozym | Rückbleibende | | Absorptionsspektren | | | | Lytische Aktivität4) |
|---|---|---|---|---|---|---|---|---|---|
| | | | $NH_2$-Gruppe# | Tyrosyl-u. Histidyl-Gruppe | $\lambda_{max}$ | $\lambda_{min}$ | $\varepsilon_{max}$ | $\varepsilon_{min}$ | |
| 1 | 12 Stdn. | — | 86 | 100 | 283 | 250 | 44.8 | 17.2 | 100% |
| 2 | | — | 100 | 〃 | 〃 | | | | 80 |
| 1 | 2 Tage | 9 | 25 | 100 | 〃 | 〃 | 〃 | 18.0 | 100 |
| 2 | | — | 91 | 〃 | | | | | 53 |
| 1 | 7 Tage | 12 | 0 | 95 | 〃 | 〃 | 〃 | 19.2 | 100 |
| 2 | | — | 76 | 100 | | | | | 7 |

* Die Enzymlösung enthält 6.42 mg Protein/ml.

** Die Lösung enthält 60 mg ÄO/ml.

# Es wurde mittels Ninhydrins bestimmt.

## Es wurde danks Folinsches Phenol-reagens bestimmt.

(nach Biuret), dann mit NaOH auf pH 8.7 eingestellt.

Zu 10 ml dieser Lysozymlösung wurde 5 ml des beinahe 40–60 mg/ml Äthylenoxyd (ÄO) haltenden Wassers zugesetzt, dann mit 1 ml von $MgCl_2$Lösung versetzt, die 100 mg/ml $Mg^{\cdot\cdot}$ enthielt und vorher auf pH 8.7 eingestellt worden war (Tabelle I). Das Reaktionsgemisch wurde bei 4–5 C unter gelegentlicher Pipettierung gelassen. Die ausgezogene Lösung in Gebrauch nehmend, bestimmte man die Menge von verbundenem ÄO, die lytische Aktivität und die freie $NH_2$-Gruppe.

Wie man aus der Tabelle I erkannt, verbanden 12 Mol von ÄO sich bei pH 8.7 mit 1 Mol vom Lysozyme und wurde die freie $NH_2$-Gruppe des Lysozyms völlig einwickelt, aber die anfängliche Aktivität blieb unverändert, während ein ziemlich grosser Abstand sich hinsichtlich der Veränderung von der Aktivität im Verlaufe der Zeit zwischen dem mit ÄO behandelten Lysozyme und nativem Lysozyme befand (Figur 2). Bewahrt man es ohne Zusatz von Mg-Verbindung während verhältnissmässig längerer Dauer auf, so reagiert ÄO ein wenig auf $NH_2$-Gruppe und freie $NH_2$-Gruppe vermindert sich einigermassen, während seine Aktivität bei weitem abnimmt. Also ist Mg-Salz sowohl ein Katalysator bei der Reaktion des ÄO, als auch spielt es für Beständigkeit vom Lysozyme eine wichtige Rolle.

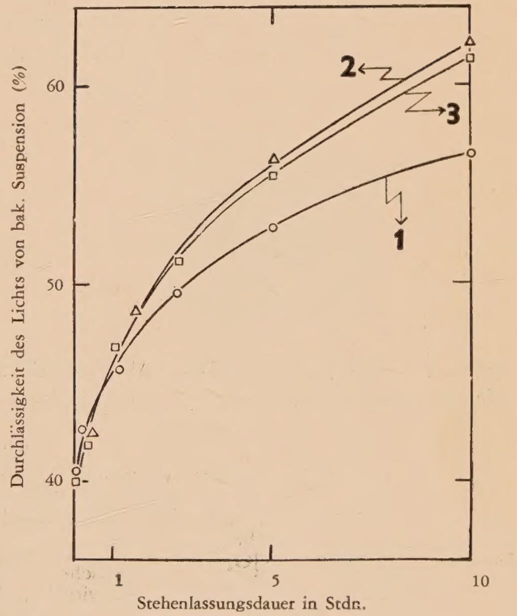
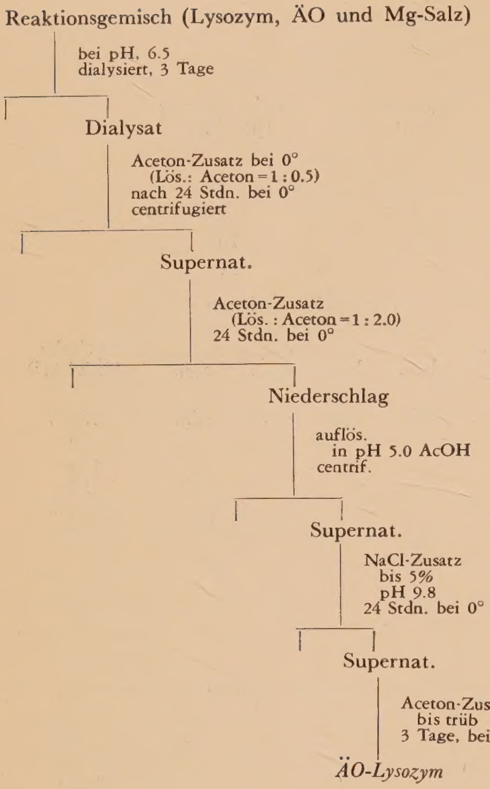


FIG. 2. Veränderung von der Aktivität im Verlaufe der Zeit bei dem mit ÄO behandelten Lysozyme.

1. ÄO-Lysozym (mit $Mg^{\cdot\cdot}$) 6.975r Enzym
2. Natives Lysozym (mit $Mg^{\cdot\cdot}$) 6.965r 〃
3. Lysozym und AO (ohne $Mg^{\cdot\cdot}$) 100r 〃



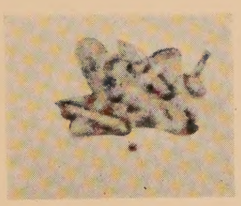


FIG. 3. ÄO-Lysozym (lytische Aktivität 100%, freie $NH_2$-Gruppe gedeckt).

*Isolierung des mit ÄO behandelten Lysozyms.*

Die mit ÄO nach oben erwähnter Weise behandelte Lysozymlosung wurde fraktioniert, wie folgendes Schema zeigt, um modifiziertes Lysozym zu isolieren. Schliesslich konnten wir mit geringer Ausbeute das Lysozym-Derivate erhalten.

**(b) Oxydation des mit ÄO behandelten Lysozyms durch $NaJO_4$.** Das modifizierte Lysozym, in welchem nicht mehr die freie $NH_2$-Gruppe vorhanden war und welches doch völlige Aktivität besass, wurde der Perjodat-Oxydation unterworfen.

Aus dem genug reagierten Gemische (2 Wochen lang stehen gelassen) wurde das überschüssige ÄO durch Lüftung beseitigt, dann wurde die Lösung mit verd. AcOH wieder auf pH 5.3 eingestellt. Zu 10 ml von dieser Enzymlösung, nach Abkühlen bis 0 C, setzte man die berechnete Menge von abgekühlter 0.05 N $NaJO_4$ Lösung (0.1 N Acetat-Puffer von pH 5.3) zu. Das Reaktionsgemisch wurde bei 4–5 C stehen gelassen. Nachdem Perjodat, nötigenfalls vermittels Hinzufügens von wenigem Traubenzucker, reduziert worden war, wurde die Lösung mit Kochsalz, dessen Konzentration bis 5%, dann mit verd. Natronlauge versetzt, um Enzyme niederzuschlagen. Der dabei entstandene Niederschlag wurde centrifugiert und mit der 5% iges Kochsalz enthaltenden, abgekühlten verd. Natronlauge von pH 9.5 wiederholt gewaschen, bis es von Halogen frei wurde, und zuletzt wieder in verd. Essigsäure von pH 5.4 aufgelöst. Mit der dabei erhaltenen Lösung mass man die enzymatiche Aktivität und das Absorptionsspektrum.

Die Tabelle II gibt die dabei erhaltenen Ergebnisse an. Aus dieser Tabelle ist zu ersehen, dass die mit ÄO behandelte Probe sich bei der Oxydation durch $NaJO_4$ ganz ähnlich mit dem Resultate über natives Lysozym benimmt. Das Verschwinden der Aktivität und die Verschiebung von UV-Absorptionsspektren standen auch jede für sich in näherer Beziehung; es befindet sich nämlich wesentlicher Unterschied nicht.

TABELLE II

OXYDATION DES MIT ÄO BEHANDELTEN LYSOZYMS MIT PERJODATE

| Versuchs Nr. | Lysozym od. sein Derivat. | Nach 8 stdg. Oxydation | | | Nach 22 stdg. Oxydation | | |
|---|---|---|---|---|---|---|---|
| | | Verbr. $NaJO_4$ Mol / Mol Lysozym | Lytische Aktivität | Extinktion beim Gipfel von 280.5mμ ($\varepsilon \times 10^{-4}$) | Verbr. $NaJO_4$ Mol / Mol Lysozym | Lytische Aktivität | Extinktion beim Gipfel von 280.5mμ ($\varepsilon \times 10^{-4}$) |
| 1 | ÄO-Lysozym | 3.1* | 43% | 2.5 | 7.8 | 3.4% | 0.85 |
| 3 | natives Lysozym | 3.3 | 43% | 2.5 | 7.8 | 3.8 | 0.85 |

* Zunahme von Mol. Gew. wurde in Betracht gezogen.

Man kann also bei der Perjodat-Oxydation keinen Einfluss wegen der Verbindung von ÄO erkennen.

## DISKUSSION

Da die Aminogruppe bei pH 8.7 zum Teile als $NH_2$ vorhanden ist, musste ÄO auf diesem Teile reagieren. Es ist auch offenbar aus der Farbreaktion mittels Phenol-reagens, dass ÄO zur Oxygruppe von Tyrosine nichts bedeutet. Es lässt sich aus der vorhergehenden Mitteilung folgern, dass ÄO mit aliphatischer Oxygruppe nichts zu tun hat.

Sowohl unser Versuch, dass freie $NH_2$-Gruppe nicht mehr erkennbar ist und doch volle Aktivität aufbewahrt worden ist, als auch das Ergebnis von Schroeder[1] liessen uns zu dem Meinung kommen, dass freie $NH_2$-Gruppe von Lysozyme mit seiner Aktivität in keiner Beziehung stehe[5].

Wenn -$CH_2CH_2OH$ anstatt H von $NH_2$-Gruppe sich verband, so muss die Glykolspaltung von den Oxyaminosäuren schwierig sein. Jedoch wurde wirklich das mit ÄO behandelte Lysozym in gleichem Benehmen mit nativem Lysozyme durch $NaJO_4$ oxydiert und seine Aktivität verlor.

Es lässt sich also erkennen, dass nicht nur $NH_2$-Gruppe, sondern auch die Oxygruppe von ihrem benachbarten Kohlenstoffe mit der Aktivität nichts zt tun haben.

## ZUSAMMENFASSUNG

Lysozym wurde in Anwesenheit von Magnesiumsalz bei pH 8.7 mit ÄO behandelt. Dadurch liess sich angeben, dass 12 Mol von ÄO sich mit pro Mol Lysozyme verbanden, freie $NH_2$-Gruppe völlig entschwand, aber die Aktivität und das Spektrum die keine merkliche Änderung zeigten. Im Falle, dass das mit ÄO behandelte Lysozym durch $NaJO_4$ oxydiert wurde, verhielt es sich mit dem nativen Lysozyme analogisch.

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[5] H. Neurath und K. Bailey; The Proteins, Vol. II. S. 465 (1954).

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 3, p. 110～115, 1956]

# Tyramine, as an Active Factor for the Production of Phenolsulphatase by *Aerobacter aerogenes*

By Tokuya HARADA and Fumio HATTORI

*Institute of Scientific and Industrial Research, Osaka Univ.*

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An active factor for the production of phenolsulphatase by some strains of *Aerobacter aerogenes* was separated as pure crystals from a commercial peptone preparation by ion exchange chromatographic techniques. Properties of this substance were studied, and it was found to be identical with tyramine. Even when pure tyramine in a concentration of $1\times10^{-5}$M was added to the culture medium, its effect on the level of enzymatic activity could be observed. In addition to tyramine, hordenine was also found effective but this, only in the case of *A. aerogenes* strain 9621. This specific activity of tyramine appears to be an induction of enzyme production.

It is only in recent years that the bacterial phenolsulphatase has been studied. The occurence of phenolsulphatase (hereafter referred to as PSase) in some strains of *Aerobacter aerogenes* was demonstrated by the authors,[1,2] and its characteristics as an enzyme in the cell-free state obtained from *A. aerogenes* strain 9621, were studied.[3] In the latter report, we demonstrated that the rate of enzyme production varied with the preparations of the peptone used in the medium. Such interesting findings lead us to suggest the existence of an active factor in some peptone preparations for the production of PSase. In fact, we found that an active factor is present in yeast extract and some commercial peptone preparations especially rich in Mikuni peptone.[4] Furthermore, we made an attempt to purify the active factor and obtained a fraction, which showed activity ten times stronger than that of the original substance.[5] In the present paper, it is described that the active factor is obtained in the crystalline state upon purification by the ion exchange chromatographic technique and that the factor proved to be identical with tyramine.

## METHODS

**Strains.** The strains of *A. aerogenes* employed in this work were: 9621, 8329 ATCC (obtained from the American Type Culture Collection through Dr. F. F. Weiss), F 50 (obtained from one of the authors (T.H.)). The organism mainly used was *A. aerogenes* 9621.

**Stock Cultures.** The various strains were maintained on agar slants composed of one per cent Mikuni peptone, 0.5 per cent yeast extract, 2 per cent agar-agar, pH 7.0. Following a monthly transfer, the cultures were incubated at 34°C for 48 hours and then stored at room temperature.

**Culture and Determination of Enzyme Activity.** The culture for inoculum was prepared by transferring one loop from the agar stab to 10 ml of broth. A twenty four hour-culture in this medium was used dropwise to inoculate each tube. The composition of the basal medium employed, is shown in Table I. Glucose should not be employed because it markedly decreased the level of enzymatic activity in the culture medium. Culture medium after seventy hour's incubation at 34°C was employed as the enzyme solution, except otherwise noted. PSase activity, on the

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[1] T. Harada, *J. Agr. Chem. Soc. Japan*, **26**, 29 (1952).

[2] T. Harada, K. Kono, and K. Yagi, *J. Agr. Chem. Soc. Japan*, **28**, 337 (1954).

[3] T. Harada and K. Kono, *J. Agr. Chem. Soc. Japan*, **28**, 608 (1954).

[4] T. Harada and K. Kono, *J. Agr. Chem. Soc. Japan*, **29**, 57 (1955).

[5] T. Harada, *J. Agr. Chem. Soc. Japan*, **29**, 874 (1955).

TABLE I

BASAL MEDIUM

| Constituent | mg, per 10 ml of the final medium |
|---|---|
| Bacto casamino acid | 50 |
| $KH_2PO_4$ | 5 |
| $(NH_4)_2HPO_4$ | 10 |
| $MgSO_4 \cdot 7H_2O$ | 2 |
| $FeSO_4 \cdot 7H_2O$ | 0.1 |
| $MnSO_4 \cdot 4H_2O$ | 0.1 |
| NaCl | 0.1 |

basis of the formation of *p*-nitrophenol, was determined by the following method. Tubes containing 2 ml of the culture medium and 1 ml of 1 M acetate buffer solution of pH 7.0, were incubated for five minutes at 34°C. After the preliminary incubation period, 1 ml of 0.01 M *p*-nitrophenyl sulphate was added to each tube so as to give a final volume of 4.0 ml. The tubes were shaked thoroughly, and incubated further under occasional shaking for one hour at 34°C. The amounts of *p*-nitrophenol produced were estimated according to the method already mentioned. A linear relationship was obtained between the rate of hydrolysis of the substrate and the incubation period within one hour. The experimental values of PSase activity were corrected for basal medium controls incubated with *A. aerogenes* 9621. Under the experimental conditions employed, *p*-nitrophenyl sulphate was not hydrolysed in the absence of the active factor by this strain. Turbidity of the culture medium was determined by a Kotaki AKA photoelectric colorimeter using filter S-66. Two ml of the culture medium which showed turbidity equivalent to an optical density of 0.350, contained approximately one mg of the dried cells.

**Chemicals.** All chemical compounds used as nutrients were of reagent grade. Amino acids were purchased from Ajinomoto Co.; tyramine hydrochloride, from L. Light and Co., Ltd; hordenine hydrosulphate, from Merck and Co., Inc; phenylethylamine and tryptamine hydrochloride, from Tokyo Kasei Co.; histamine hydrochloride and methylamine hydrochloride, from Wako Pure Chem. Ind., Ltd. Bacto casamino acid was used as a casein-hydrolysate. *p*-Nitrophenyl sulphate as an enzyme substrate was synthesized according to the modified methods of Whitehead et al.[6].

## RESULTS

**Ion Exchange Chromatography of Active Factor.** As reported previously,[6] Mikuni peptone was extracted with hot 95% ethanol. The extract was absorbed on active charcoal and then eluted with a mixture of phenol, acetic acid, and water (4:1:15, by volume). The active fraction thus obtained, was named the MB fraction. Paper chromatography of MB fraction with pyridine-water (4:1, by volume) as a developing solvent, revealed that the active substance accumlated at $R_F$ 0.84 had a strong violet spot with ninhydrin reagent. Because none of the known amino acids and $R_F$ value in the neighbourhood of 0.84, it was supposed that the active substance might be a peptide. So, we have attempted to separate the active substance from MB fraction by applying ion exchange chromatography, which is known to be an effective method for separation of peptides.[7] A sample of MB fraction was prepared for chromatography as follows: One hundred mg of MB fraction was dissolved in 4 ml of pH 4.25 acetate buffer and 6 N hydrochloric acid was added to it until the pH of the solution became 1.4. This solution was then poured into the column. The dimensions of the column packed with Dowex-50 were 1×100 cm. The flow rate of the developer through the column, was adjusted to about 10 ml per hour and approximately 1 ml fractions were collected by an automatic fraction collector. As an eluting solution, acetate and carbonate buffers were used. It may be noted that phosphate or citrate buffers should not be used, since these buffers inhibit PSase activity. One ml aliquots of the fractions were neutralized with 0.1 N hydrochloric acid and diluted to 3.5 ml. Two ml portions of this solution were added to the basal medium and the PSase activity of these culture medium on incubation with *A. aerogenes* 9621, was then determined. Activity was not observed when fractions were collected from the column with the acetate buffer of pH 4.25 and 6.7, and also with the sodium bicarbonate buffer of pH

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[6] J.E.M. Whitehead, A.R. Morrison and L, Young, *Biochem. J.*, **51**, 585 (1952).

[7] S. Moore and W.H. Stein, *J. Biol. Chem.*, **190**, 103 (1951)

9.2 and 0.1 M sodium carbonate solution. Activity was deternined when eluted with 0.1 M sodium hydroxide. Therefore, after changing the eluent to this solution, 0.3 ml portions of 3.5 ml of the neutralized solution were analyzed by the modification of the ninhydrin method as described by Moore and Stein.8) The results are shown in Fig. 1.

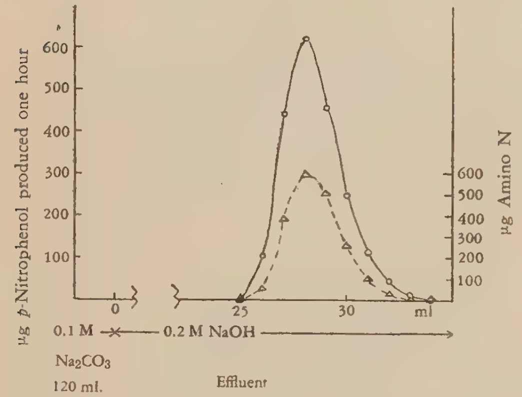

FIG. 1. Chromatography of Active Factor in MB Fraction by Dowex-50.

—○—○—○— µg $p$-Nitrophenol
··△··△··△·· µg Amino N

From these results it was found that the active factor emerges when a solution of 0.2 M sodium hydroxide is used after passage of a solution of 0.1 M sodium carbonate. The rate of PSase producing activity developed in a series of fractions, was found to be proportional to the intensity of coloration produced with the ninhydrin reagent. When a small portion of the ninhydrin-positive fraction was chromatographed on a paper strip with pyridine-water (4:1) as the developing solvent, a single violet spot at 0.84 was shown. A similar result was obtained from Mikuni peptone. On the other hand, Bacto peptone, which exhibits very weak PSase producing activity, gave neither significant PSase producing substance nor ninhydrin-positive fraction of $R_F$ 0.84. These results demonstrate the fact that a substance which gives a ninhydrin positive spot might be an active factor.

**Chromatographic Isolation of the Active Factor.** In these experiments, Mikuni peptone was subjected to chromatography. In order to handle a large amount of the substances, a Dowex-50 column (4×100 cm) was used. The Na form of this resin was buffered with pH 6.7 acetate buffer. One hundred grams of Mikuni peptone dissolved in 2 l of the same buffer was added into the column and eluted with 0.1 M sodium carbonate solution until the coloration with ninhydrin reagent was no longer exhibited. When this coloration became negligible, the eluent was changed to 0.2 M sodium hydroxide solution. Fractions of 5 ml were collected. Consequently, a small portion of each fraction was neutralized with hydrochloric acid and the neutral solution tested with the ninhydrin reagent. The faint violet color which appeared at fraction 83, became maximum at fraction 97 and almost diminished at fraction 135. Possibility of contamination by free amino acids would be avoided because they had been eluted far before this ninhydrin-positive substance was eluted. All of the ninhydrin-positive fractions showed a single spot at $R_F$ 0.84 by the method mentioned above. Thus, about 100 ml of the solution, obtained by combining fractions 83 to 130, was neutralized to pH 6.2 with conc. hydrochloric acid.

**Crystallization.** To about 100 ml of the fracion containing the active factor 20 g of the activated charcoal treated beforehand, with 5% acetic acid solution was added. The mixture was stirred vigorously for one hour and filtered. The wet charcoal was then mixed with 500ml of a mixture of methanol-acetic acid-water (2:2:1) and the whole was stirred for one hour. After removing the charcoal, the eluate was evaporated to dryness under reduced pressure. The yield of the dried substance was about 500 mg. This was dissolved in a minimum amount of hot water then cooled in an ice box at 5°C. The crude

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8) S. Moore and W.H. Stein, *J. Biol. Chem.*, **176**, 367 (1948).

crystalline substance thus obtained, was recrystallized repeatedly in the same way. The average yield of the recrystallized active factor from each 100 g of Mikuni peptone was about 200 mg.

**The Identification of the Active Substance as Tyramine.** The properties of this crystalline substance were found to be as follows: It is soluble in water, ethanol and methanol containing water, but insoluble or very slightly soluble in various other organic solvents such as acetone, benzene, toluene, ether and chloroform. It gives a reaction positive to Mörner, diazo, Millon and nitroso naphthol reagents and negative to Biuret, Lieberman and Rhode reagents. Its absorption spectrum shows a maximum at 274–278 m$\mu$. These facts indicate that the substance contains a phenolic and an amino group. It does not decompose by treatment with conc. hydrochloric acid at 15 lb. pressure for twenty-four hours. It gives a spot at $R_F$ 0.84 with pyridine-water solvent (4 : 1) and 0.62 with butanol-acetic acid-water solvent (4 : 1 : 5). These $R_F$ values are found to be identical with those of tyramine. It melts at 263–264°C (uncorr.) and at 263–265°C when mixed with the authentic tyramine hydrochloride. *Anal.* Cald for $C_8H_{11}NO \cdot HCl$: C, 55.28 ; H, 7.49 ; N, 8.06. Found : C, 54.96 ; H, 6.90 ; N, 8.42.

From these results, it was confirmed that this crystalline substance is identical with tyramine hydrochloride.

**Specific Activity of Tyramine.** In addition to tyramine, a number of tyramine related compounds were tested in respect of their effect on the production of PSase. Each substance was added at a concentration of $3 \times 10^{-3}$ M aseptically to the basal medium. *A. aerogenes* 9621 cells were grown for seventy hours, then the PSase activity of the culture medium was determined according to the procedures previously described. When activity was not observed within the specified time, incubation time was extended to six days. Data obtained with the typical experiment are shown in Table II. It was found that both the culture media supplemented with tyramine or hordenine exhibited activity, although hordenine was found less effective than tyramine. Hordenine was shown to be effective after six days' culture incubation. The following compounds were found to be inactive even after six days' incubation, under the above experimental conditions : tyrosine, phenylethylamine, tryptamine, histamine, methylamine. All the other 17 amino acids tested were found inactive under the same conditions.

TABLE II

EFFECT OF TYRAMINE AND ITS RELATED COMPOUNDS INCORPORATED IN THE CULTURE MEDIUM ON PSASE ACTIVITY

| Supplements | Turbidity of culture medium O. D. | Sulphatase activity $\mu$g N. P. |
|---|---|---|
| Tyramine | 0.44 | 76 |
| Tyrosine | 0.41 | 0 |
| Hordenine* | 0.26 | 50 |
| Phenylethylamine | 0.35 | 0 |
| Tryptamine** | 0.23 | 0 |
| Histamine | 0.23 | 0 |
| Methylamine | 0.38 | 0 |
| No addition | 0.38 | 0 |

$\mu$g *p*-Nitrophenol produced in the course of one hour in a reaction mixture containing 2ml of the 70 hour-culture medium to which a supplement had been added to a concentration of $3 \times 10^{-3}$M.

* Incubation period of culture was 6 days.

** $1 \times 10^{-3}$M added.

In order to ascertain whether a similar effect would be obtained by all the PSase positive strains of *A. aerogenes*, three strains were examined (Table III). Each of these strains increased the level of enzymatic activity markedly, when tyramine was added. Hordenine exhibited production of PSase exclusively for *A. aerogenes* 9621. In this case, the proportion of the production of PSase was larger after six and nine days than after seventy hours. This fact led us to postulate that hordenine might be converted into tyramine during the incubation period. The effect of tyramine was shown to be proportional to the amount of the compound as shown in Table IV. Even in a very dilute concent-

TABLE III

EFFECT OF TYRAMINE AND HORDENINE INCORPORATED IN THE CULTURE MEDIUM ON PSASE ACTIVITY OF THREE STRAINS OF *AEROBACTER AEROGENES*

| Strain | Incubation period of culture days | No addition | | Tyramine | | Hordenine | |
|---|---|---|---|---|---|---|---|
| | | O. D. | μg N. P. | O. D. | μg N. P. | O. D. | μg N. P. |
| ATCC 9621 | 3 | 0.36 | 0 | 0.42 | 75 | 0.24 | 0 |
| | 6 | 0.37 | 0 | 0.45 | 202 | 0.26 | 51 |
| | 9 | 0.37 | 0 | 0.44 | 330 | 0.32 | 348 |
| ATCC 8329 | 3 | 0.26 | 0 | 0.44 | 22 | 0.24 | 0 |
| | 6 | 0.32 | 0 | 0.44 | 72 | 0.24 | 0 |
| | 9 | 0.33 | 0 | 0.52 | 90 | 0.23 | 0 |
| F 50 | 3 | 0.44 | 3 | 0.47 | 90 | 0.36 | 1 |
| | 6 | 0.45 | 15 | 0.56 | 272 | 0.37 | 8 |
| | 9 | 0.43 | 27 | 0.54 | 325 | 0.37 | 15 |

μg *p*-Nitrophenol produced in the course of one hour in a reaction mixture containing 2ml of a time varying culture medium, to which the respective supplement had been added to a concentration of $3\times10^{-3}$M.

ration such as $10^{-5}$ M of tyramine, the effect was detectible. Therefore, it would be suggested that the effect of tyramine might be catalytic as in the case with vitamins.

TABLE IV

EFFECT OF CONCENTRATION OF TYRAMINE INCORPORATED IN THE CULTURE MEDIUM, ON PSASE ACTIVITY

| Molar concentration of tyramine | *Aerobacter aerogenes* strain 9621 O. D. | μg N. P. | strain F 50 O. D. | μg N. P. |
|---|---|---|---|---|
| 0 | 0.38 | 0 | 0.41 | 6 |
| $1\times10^{-5}$ | 0.38 | 1 | 0.41 | 10 |
| $3\times10^{-5}$ | 0.38 | 5 | 0.41 | 15 |
| $1\times10^{-4}$ | 0.38 | 13 | 0.41 | 19 |
| $3\times10^{-4}$ | 0.38 | 25 | 0.41 | 31 |
| $1\times10^{-3}$ | 0.40 | 69 | 0.45 | 63 |
| $3\times10^{-3}$ | 0.44 | 88 | 0.49 | 71 |

μg *p*-Nitrophenol produced in the course of one hour in a reaction mixture containing 2ml of 70 hour-culture medium to which had been added tyramine in various concentrations.

These experiments were made to clarify whether tyramine is an activator for the inert enzyme or not. In these experiments acetone dried-preparations of *A. aerogenes* 9621 on culture medium with or without tyramine, were used as the enzyme preparations. The former cells were termed T-cells and the latter B-cells. The results are shown in Table V. The reaction mixture containing the B-cells exhibited no activity even when incubated with tyramine or with heated T-cells. This fact suggests that the increase of enzymatic activity is not due to the activation of a preformed but an inert enzyme by tyramine or a derived compound from tyramine.

TABLE V

PHENOLSULPHATASE ACTIVITIES OF ACETONE TREATED CELLS

| | μg N. P. |
|---|---|
| 3 mg B-cells | 0 |
| 〃 + $1\times10^{-3}$M tyramine | 0 |
| 〃 + 3 mg heated T-cells | 0 |
| 〃 + 3 mg unheated T-cells | 55 |
| 3 mg T-cells | 57 |

μg *p*-Nitrophenol produced in the course of one hour in a reaction mixture containing acetone powders of cells, which had been cultured for 70 hrs.

B-cells, cultured on basal medium.

T-cells, cultured on basal medium supplemented by $3\times10^{-3}$M tyramine.

However, it has not yet been clarified whether the active factor has concerned with the net enzyme synthesis or not. Further studies on the role of tyramine in the production of PSase are now in progress.

## DISCUSSION

In our previous paper, it was reported that the level of PSase activity of culture medium which was incubated with some strains of *A. aerogenes*, *Salm. shottmuelleri*, or *Salm. typhi*, varied a great deal, depending upon the peptone preparations applied to the medium. When Bacto peptone was used, no significant activity was observed. Moreover, no PSase activity was exhibited by *A. aerogenes* 9621 in synthetic media containing vitamins, amino acids, purine, pyrimidine compounds and inorganic salts under the experimental conditions commonly used. However, Mikuni peptone resulted in a remarkable increase of the level of PSase activity with all the strains tested. Our present paper shows that an active principle, which is contained in Mikuni peptone, is tyramine. It also appears that no other active factors are to be contained in Mikuni peptone as those indicated by our chromatographic studies. It has been shown that the rate of activity on the culture medium containing 0.05% Mikuni peptone is almost the same as that on the medium supplemented with 0.0005% tyramine and that tyramine can be isolated from Mikuni peptone, in a yield about 0.5%.

The presence of tyramine in casein hydrolyzate prepared with toluene as an antiseptic, was indicated by Fukuda[9] from the results obtained by the paper chromatographic method of Urbach.[10] We detected the PSase-producing activity in 10% casein hydrolyzate solutions by the application of various hydrolyzing agents such as pancreatin or takadiastase. From these studies, the active factor in other peptone preparations, also appears to be tyramine.

On the other hand, it was reported by the authors[4] that active factors are found in yeast extract, "hens' liver powder" and "liver and stomach powder". Tyramine might be present in these samples. In fact, YB fraction, a concentrate of the active factor from yeast extract showed the activity of PSase production and ninhydrin-positive spot in the neighbourhood of $R_F$ value of tyramine.

Bacterial PSase has been also found in *Streptococcus alcalophilus*, staphylococci, several species of *Salmonella*, several "cold blooded" species of *Mycobacterium*, *Alcaligenes metalcaligenes* and gingival bacteria, by one of the authors[2,11,12] (T.H.), Barber et al.[13], Ishikawa[14], Whitehead et al.[6], Dodgson et al.[15] and Schultz-Hardt et al.[16] These PSase, with the exception of that of gingival bacteria, have been found to be produced non-adaptively in substrate of enzyme although this is not known with respect to staphylococci. Also, observation revealing that the rate of enzyme production varied with composition of the medium, has been made with *Str. alcalophilus*[17] and the gingival bacteria.[16]

However, the mechanism of PSase production by any of these bacteria still remains obscure. The effect of tyramine on the PSase production of species other than *A. aerogenes*, must be studied. The studies on the role of tyramine for the production of PSase is of special interest, since tyramine is known to be the active substance responsible for the metabolism of higher animals.

The authors wish to express their sincere thanks to Prof. Z. Nikuni for his kind advice and suggestions rendered throughout the course of this study and to Mr. M. Yoshida for his assistance. They also wish to thank Mr. T. Fujino for the spectrophotometric analysis.

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[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 3, p. 116~121, 1956]

# Studies on the Carbohydrates of Horse Radish, *Cochlearis Armoracia* L.

## Part I. The Carbohydrates in the Root. (1).

By Takashi MIZUNO and Kaneo HAYASHI*

*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University*

Received April 18, 1956

In the root of horse radish, sucrose, fructose, glucose and two unknown fructose-oligosaccharides were found in the form of free sugars, in the decreasing order just given, and large amounts of starch, hot 50% methanol-soluble polysaccharide, water-soluble polysaccharide, pectin, hemicellulose, cellulose and a small amount of lignin were found as polysaccharides. As the constructive sugar of these polysaccharides, the following, i.e., glucose, fructose, galactose, arabinose, galacturonic acid and xylose were detected.

In recent years, the cultivation of horse radish (*Cochlearis Armoracia* L.) as a substitute for "Wasabi" (*Eutrema Wasabi* Maxim.) has been extensively carried out in the cool regions of this country, with a fairly large production.

In view of the fact that practically only a few chemical investigations had been made,[1, 2, 3] comprehensive studies on the horse radish were projected. To begin with, this report was made on the basis of some of our studies obtained about the carbohydrate, the main component of the root of horse radish.

### EXPERIMENTAL

### I. Preparation of the Powdered Sample.

The root of horse radish, grown in Nagano-ken and Hokkaido harvested from November to December 1953, was washed, cut, dried by heating (below 70°, within 5 hours) and pulverized so as to pass a 1-mm sieve.

### II. General Chemical Composition of the Root.

The results of analysis based on the established method are those shown in Table I.

TABLE I

CHEMICAL COMPONENTS OF THE HORSE RADISH ROOT (% FOR DRY BASIS)

| | | | |
|---|---|---|---|
| Crude Ash | $5.2_0$ | Crude Fiber | 3.73 |
| Crude Protein | 12.09 | N-free Extract | 77.98 |
| True Protein[*1] | 5.76 | Pentosan[*2] | 10.06 |
| Non-protein Nitrogen | 0.99 | | |
| Crude Fat | 1.00 | (Moisture of Fresh root 78.31%) | |

[*1] By Barnstein's method.

[*2] By Tollens-Sasaki-Schmidt's method.

This table indicates that the main component of horse radish was carbohydrate.

### III. Fractionation and Identification of Carbohydrates.[4,5,6]

After the powdered sample had been dried for 3 hours at a temperature below 70°, the fractional extraction of carbohydrate was proceeded with the various solvents and fifteen fractions were obtained, as shown in Fig. 1.

The extraction was repeated from three to six times with the same solvent until Molisch's reaction of the extract became negative; after the residue was sufficiently washed with the same solvent, dried, and weighed, it was put through the following extractive

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* Department of Agricultural Chemistry, Faculty of Agriculture, Gifu University.

[1] Sasaki: *J. Agr. Chem. Soc. Japan*, **2**, 406 (1926).

[2] A. Heldueschka und A. Zwergal: *J. Prakt. Chem.*, **132**, 201 (1932).

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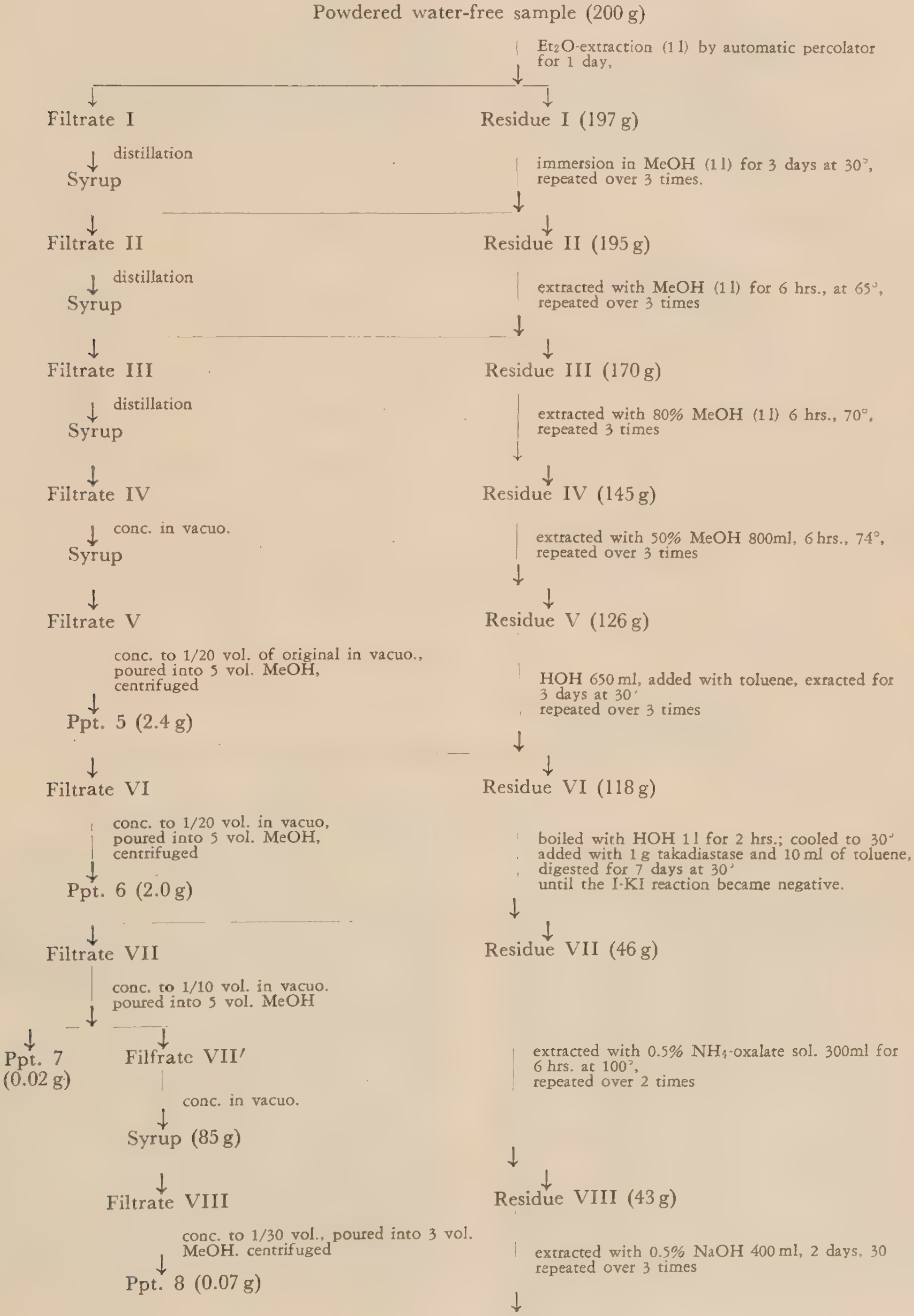
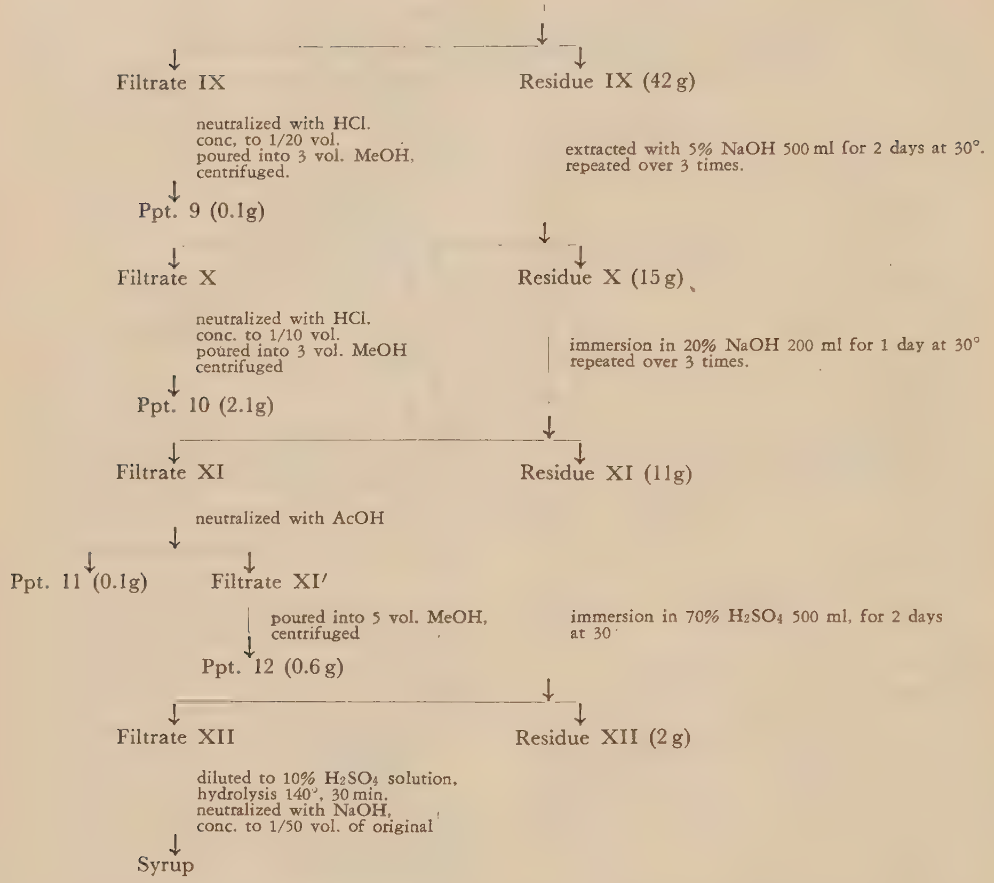
[6] T. Mizuno and T. Kinpyô: ibid., **29**, 848 (1955).

FIG. 1. The Fractionation of Carbohydrates in Horse Radish Root.


Powdered water-free sample (200 g)
Et₂O-extraction (1 l) by automatic percolator for 1 day,
Filtrate I
distillation
Syrup
Residue I (197 g)
immersion in MeOH (1 l) for 3 days at 30°, repeated over 3 times.
Filtrate II
distillation
Syrup
Residue II (195 g)
extracted with MeOH (1 l) for 6 hrs., at 65°, repeated over 3 times
Filtrate III
distillation
Syrup
Residue III (170 g)
extracted with 80% MeOH (1 l) 6 hrs., 70°, repeated 3 times
Filtrate IV
conc. in vacuo.
Syrup
Residue IV (145 g)
extracted with 50% MeOH 800 ml, 6 hrs., 74°, repeated over 3 times
Filtrate V
conc. to 1/20 vol. of original in vacuo., poured into 5 vol. MeOH, centrifuged
Ppt. 5 (2.4 g)
Residue V (126 g)
HOH 650 ml, added with toluene, exracted for 3 days at 30° repeated over 3 times
Filtrate VI
conc. to 1/20 vol. in vacuo, poured into 5 vol. MeOH, centrifuged
Ppt. 6 (2.0 g)
Residue VI (118 g)
boiled with HOH 1 l for 2 hrs.; cooled to 30° added with 1 g takadiastase and 10 ml of toluene, digested for 7 days at 30° until the I-KI reaction became negative.
Filtrate VII
conc. to 1/10 vol. in vacuo. poured into 5 vol. MeOH
Ppt. 7 (0.02 g)
Filfrate VII′
conc. in vacuo.
Syrup (85 g)
Residue VII (46 g)
extracted with 0.5% NH₄-oxalate sol. 300 ml for 6 hrs. at 100°, repeated over 2 times
Filtrate VIII
conc. to 1/30 vol., poured into 3 vol. MeOH. centrifuged
Ppt. 8 (0.07 g)
Residue VIII (43 g)
extracted with 0.5% NaOH 400 ml, 2 days, 30 repeated over 3 times



operations.

Filtrate I–IV were divided into two portions respectively. One portion was concentrated in vacuo at 70°, and was submitted to paper chromatography. And the other was evaporated, made to 0.2% sulfuric acid solution and hydrolysed for 30 minutes in the boiling-water bath. The resulting solution was neutralized with NaOH solution or powdered CaCO$_3$, further concentrated to dryness under reduced pressure; the solid obtained was extracted two or three times with warm 80% methanol and the alcoholic extract evaporated to the syrup. This syrup was examined by paper chromatography[4,5,6] in order to investigate the component sugars of polysaccharide.

The carbohydrates in the fraction of filtrate V–XI existed in the form of polysaccharide. After the addition of methanol to the concentrated extract, the precipitates produced were powdered and then the constructive sugars of these polysaccharides were investigated by the following method.

After the alkaline solution was neutralized with sulfuric acid, hydrochloric acid or acetic acid, each filtrate was concentrated under reduced pressure to a suitable volume (1/10–1/30 volume of the original solution), and the concentrates were thrown into three to five volumes of cold methanol to precipitate each polysaccharide.

The sediments were separated from their mother liquor by the centrifugation, and the precipitates obtained were washed with increasing concentrations of methanol from 50% to 100%. The wet polysaccharides, finally obtained, were dried in a desiccator under reduced pressure, powdered, and hydrolysed in order to investigate the constructive sugars with paper chromatography.[4,5,6]

After twenty ml of 5% H$_2$SO$_4$ or 3% HCl was added to 1 gram of the powdered polysaccharide, this mixture was subjected to hydrolysis from one to

TABLE II

Paper Chromatograms of Sugars in Each Fraction

Filter paper; Toyô No.50, 40×6 cm.
Solvent; *n*-BuOH: AcOH: HOH (4:1:2 by vol.)
Reagents; Aniline hydrogen phthalate butanol solution (A), Phloroglucinol HCl ethanol solution (B) and Ammoniacal silver nitrate ethanol solution (C).
Development method; One dimensional ascending method or Multiple ascending method. (30′)

| Sugars | | Fructose | Glucose | Galactose | Xylose | Arabinose | Galacturonic acid | Maltose | Sucrose | Unknown 1 | Unknown 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $R_F$ value | | 0.23 | 0.18 | 0.16 | 0.28 | 0.26 | 0.13 | 0.12 | 0.14 | 0.07 | 0.04 |
| Color reaction | (A) | − | + | + | + | + | + | + | − | − | − |
| | (B) | + | − | − | + | + | − | − | + | + | + |
| | (C) | + | + | + | + | + | + | + | − | − | − |
| Fractions | | | | | | | | | | | |
| Filtrate I | (a)*1 | − | − | − | − | − | − | − | − | − | − |
| | (b)*2 | − | ± | − | − | − | − | − | − | − | − |
| Filtrate II | (a) | + | + | − | − | − | − | − | + | − | − |
| | (b) | ++ | + | − | − | − | − | − | − | − | − |
| Filtrate III | (a) | + | + | − | − | − | − | − | +++ | + | + |
| | (b) | +++ | ++ | − | − | − | − | − | − | − | − |
| Filtrate IV | (a) | − | − | − | − | − | − | − | + | − | − |
| | (b) | + | + | − | − | − | − | − | − | − | − |
| Ppt. 5*3 | | − | ± | + | − | + | − | − | − | − | − |
| Ppt. 6*3 | | − | ++ | ± | − | ± | + | − | − | − | − |
| Ppt. 7*3 | | − | − | + | − | + | − | − | − | − | − |
| Filtrate VII′ | | − | +++ | − | − | − | − | ++ | − | − | − |
| Ppt. 8*3 | | − | − | ± | − | ± | ± | − | − | − | − |
| Ppt. 9*4 | | − | ± | − | ± | ± | − | − | − | − | − |
| Ppt. 10*4 | | − | + | − | ++ | − | − | − | − | − | − |
| Ppt. 11*4 | | − | + | − | + | − | − | − | − | − | − |
| Ppt. 12*4 | | − | ± | − | + | − | − | − | − | − | − |
| Filtrate XII | | − | ++ | − | ± | − | − | − | − | − | − |

+++ Very intense, ++ Intense, + Positive, ± Very weak, − Negative,

*1 Original solution being used.

*2 Original solution hydrolysed with 0.1% HCl for 30 min at 100°.

*3 Ppt. was hydrolysed with 5% $H_2SO_4$ for 1–3 hrs, at 100°.

*4 Ppt. was hydrolysed with 3% HCl for 1–3 hrs, at 100°.

three hours in the boiling-water bath, and the resulting solution was neutralized by adding powdered $CaCO_3$ or $Na_2CO_3$. The neutralized solution was evaporated and dried up under reduced pressure below 80°. The solid obtained was extracted three of four times with warm 80% methanol and the filtrates were combined. The extract was evaporated to remove methanol and the syrup was subjected to paper chromatography in parallel with the standard sugar.

## IV. Results of Identification on the Carbohydrates.

The results obtained from the above mentioned fractionation and identification are summarized in Table II, the yields of each fractions being shown in Table III.

TABLE III

Yields of Each Fraction (% as dry matter)

| Residue | (%) | Filtrate | (%) | Ppt. | (%) |
|---|---|---|---|---|---|
| I | 98.5 | I | 1.5 | | |
| II | 97.5 | II | 1.0 | | |
| III | 85.0 | III | 12.5 | | |
| IV | 72.5 | IV | 12.5 | | |
| V | 63.0 | V | 9.5 | 5 | 1.20 |
| VI | 59.0 | VI | 4.0 | 6 | 1.00 |
| VII | 23.0 | VII | 36.0 | 7 | 0.01 |
| VIII | 21.5 | VII′ | 42.5* | 8 | 0.04 |
| IX | 21.0 | VIII | 1.5 | 9 | 0.05 |
| X | 7.5 | IX | 0.5 | 10 | 1.05 |
| XI | 5.5 | X | 13.5 | 11 | 0.05 |
| XII | 1.0 | XI | 2.0 | 12 | 0.30 |
| | | XII | 4.5 | | |

* This fraction was a syrup containing ca. 25% moisture.

Explanations for the above results in each fraction were as follows:

Filtrate I: This fraction was shown as crude fat; the appearance of a trace of glucose by the acid hydrolysis lead to a suggestion that this originated from glycosides.

Filtrate II: Glucose and fructose were detected as free sugars, and a trace of sucrose was, also, found in this fraction.

Filtrate III: A large amount of sucrose, extracted with warm methanol, was detected; sucrose disappeared by the acid hydrolysis and produced glucose and fructose on the paper chromatogram. Moreover, a small amount of glucose, fructose and two unknown oligosaccharides ($R_F$ 0.07 and 0.04) were detected. The two last mentioned, were believed to be identical with the fructose-oligosaccharides, which were proved to already exist in the bulb of *Lycoris radiata* Herbert[7]), and the lower leaves of *Allium fistulosum* L.[5]) by the present authors. More minute details, however, have been left for future studies.

Filtrate IV: In this fraction obtained by extraction with hot 80% methanol, a small amount of sucrose was found but no other free sugar was detected.

Ppt. 5: Galactose, arabinose and a trace of glucose were detected as the component sugars of polysaccharide in this fraction.

It may naturally be deduced that a kind of galactan or araban existed as the lower molecular polysaccharides, since they have been extracted with the hot 50% methanol.

Ppt. 6: Galactose, arabinose and galacturonic acid were detected as the component sugar of this polysaccharide. These sugars were considered to originate from the cold water-soluble pectin.

And also, the presence of cold water-soluble glucosan in the root was suspected because a considerable amount of glucose detected in the hydrolysate of Ppt. 6.

Ppt. 7: A polysaccharide (Ppt. 7) was separated by adding methanol to the filtrate which was obtained from the digestion of Residue VI with takadiastase (Sankyô Co. products). This polysaccharide consisted of galactose and arabinose.

Filtrate VII′: Large amounts of maltose and glucose were detected in this fraction. A considerable amount of syrup (yield 43% for water-free raw material) was produced by the concentration.

Ppt. 8: The yield of the powdered Ppt. 8 was very small, but galactose, arabinose and galacturonic acid were detected as component sugars of this precipitate. It could be considered that this was a polysaccharide corresponding to the protopectic substance existing in the cell wall.

Ppt. 9: The yield of the precipitate was a small amount, and xylose and glucose were detected as the component sugars. This polysaccharide is, therefore, considered to be a kind of hemicellulose.

Ppt. 10: This was corresponding to hemicellulose also, and xylose and glucose were found in its hydrolysate. It can be deduced that there was the presence of a kind of xylan there.

Ppt. 11: This precipitate was β-cellulose, because xylose and glucose were also detected as the component sugars.

Ppt. 12: This was γ-cellulose fraction, because xylose and glucose were found in its hydrolysate.

Filtrate XII: Residue XI, crude α-cellulose, was treated with the cold 70% $H_2SO_4$ solution to dissolve the cellulose, and filtrate XII was obtained. Large amounts of glucose and a trace of xylose were detected in filtrate XII.

Residue XII: This was Klason's lignin, but it appeared that the humus substances were also present because of the drastic destruction of carbohydrates; contents of ash and $CH_3O$ were 0.28 and 9.67%, respectively.

## SUMMARY

The carbohdrates in the root of horse radish, *Cochlearis Armoracia* L., were divided into fifteen fractions (see Figure 1).

Free sugars and the component sugars in each fraction have been mainly investigated by paper chromatography and the results obtained were as follows (see also Table II).

Fructose and glucose were detected as free monosaccharides; and, sucrose and two unknown oligosaccharides consisting of fructose only, as free oligosaccharides.

Galactose, arabinose and glucose were detected as the component of the hot 50% methanol-soluble polysaccharides.

There were detected glucose, galacturonic acid, arabinose and galactose as the components of the cold water-soluble polysaccharides, galactose and arabinose as the hot water-soluble polysaccharides, respectively.

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7) T. Mizuno and K. Hayashi: ibid., **28**, 349 (1954).

Large amounts of starch (horse radish starch) and a small amount of pectin (hydrato- and proto-pectin) existed in the root of horse radish.

Xylose, glucose and arabinose were detected as the component sugars of hemicellulose.

Xylose and glucose as the component sugars of both $\beta$-and $\gamma$-cellulose fractions were found respectively. $\alpha$-cellulose and a small amount of lignin were also detected.

The authors wish to express their profound gratitude to Mr. Motoji Kobayashi, President of the Kinjirushi Wasabi Co., Ltd., to whom they are indebted for the valuable research materials and funds cordially given.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 3, p. 122～126, 1956]

# Studies on Cycasin, a New Toxic Glycoside, of *Cycas revoluta* Thunb.

## Part VI. Polarography of Cycasin

By Kotaro NISHIDA, Akira KOBAYASHI and Tomonori NAGAHAMA
*Biochemical Laboratory, Faculty of Agriculture, Kagoshima University*

Received May 7, 1956

The polarographic behaviors and the determination of cycasin, glucosyloxyazoxymethane, are described here. In the whole pH range, cycasin shows a reduction wave, which is considered to be due to the reduction of its aliphatic azoxy group. The polarograms from pH 4 to 7 run in two steps, and two electrode reaction mechanisms may be infered. The following facts are observed on the limiting current at pH 1 or 7: It is controlled by the diffusion process, and its temperature coefficient has a value resembling that of the usual diffusion current. The wave height shows a linear proportionality to the concentration of cycasin. The contents of cycasin in cycad seeds, determined polarographically, are presented, and compared with the results obtained by the paper chromatographic method.

Quantitative determination of cycasin was very difficult when it was interfered by coexisting sugars, since they could not be removed so easily. This difficulty was to some extent resolved by application of paper chromatography, as reported in the preceding paper[1]. It was, however, expected that cycasin could be determined by a more specific and simple method based upon its azoxy group[2] in the aglycone part. Thus, in the present experiment, polarographic investigations on cycasin were carried out, as a result of which an alternating method of analysis was established.

### MATERIALS AND METHODS

Cycasin (m.p. 144°–145°C decomp.), isolated from cycad seeds by the authors, was dissolved in distilled water in a concentration of $5\times10^{-3}$ mol. per liter. This stock solution was diluted to a definite volume with a buffered solution containing 0.2 N potassium chloride. The buffer solutions were prepared according to McIlvaine (pH 2–8) and Sörensen (pH 9–12), of which the pH values were determined with a glass-electrode pH meter prior to use. The solution of pH 1 or 13 was prepared by using N/10-hydrochloric acid or sodium hydroxide. The characteristics of the capillary used for the dropping mercury electrode were as follows; m, the rate of flow, was 1.795 mg sec.$^{-1}$, and t, the drop time, was 4.0 sec. ($m^{2/3}\ t^{1/6}=$ 1.860 mg$^{2/3}$ sec.$^{-1/2}$) in 0.2 N potassium chloride solution at −1.5 V vs. normal calomel electrode (N.C.E.). Throughout the experiments, N.C.E. was used as the reference electrode, the dissolved oxygen was removed by bubbling of hydrogen gas, and the temperature of the electrolytic solution kept at 25°C. The polarograph employed was a Yanagimoto pen-recording type.

### RESULTS AND DISCUSSION

**1. Effect of pH upon the Polarograms** As shown in Fig. 1, cycasin gave either a single or a two-step reduction wave at various pH values. Round maxima, which can be suppressed by the addition of gelatin, were especially markedly observed at pH 6 or 7. Table I represents the relationships between pH, the half-wave potential and the limiting current. The limiting currents from pH 1 to 4 and 9 to 11 were approximately equal, and corresponded to about two-thirds of the maximum value of the current found at pH

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[1] K. Nishida, A. Kobayashi and T. Nagahama, This Bulletin **20**, 74 (1956).

[2] *ibid*, **19**, 77 (1955).

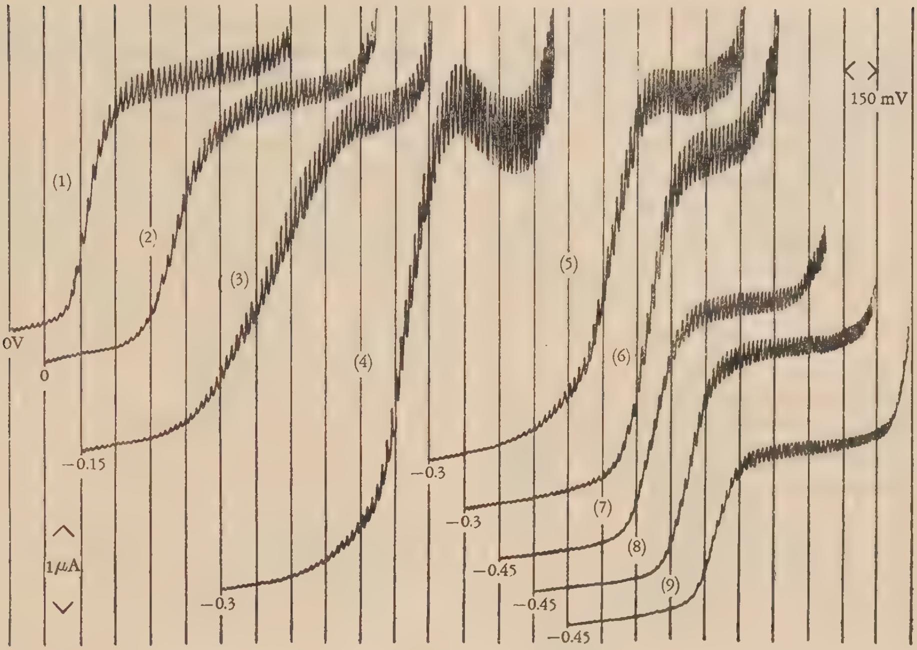


FIG. 1. Polarograms of Cycasin.

$2.5\times10^{-4}$M cycasin in 0.2 N KCl, at 25°C, at pH (1) 1.0, (2) 3.0, (3) 5.0, (4) 6.0 (gelatin non-added), (5) 6.0 (6) 7.0, (7) 9.0, (8) 11.1, (9) 13.0.

TABLE I

RELATIONSHIPS BETWEEN pH, THE HALF-WAVE POTENTIAL AND THE LIMITING CURRENT

$2.5\times10^{-4}$M Cycasin in 0.2 N KCl, Gelatin added, at 25°C

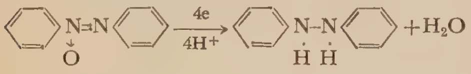
| pH | $E_{1/2}$, V vs. N.C.E. | i, μA |
|---|---|---|
| 1.0 | −0.34 | 2.59 |
| 2.1 | −0.51 | 2.59 |
| 3.0 | −0.57 | 2.67 |
| 3.9 | −0.67 | 2.70 |
| 5.0 | −0.96 | 3.55 |
| 5.9 | −1.08 | 3.83 |
| 7.0 | −1.10 | 3.64 |
| 8.1 | −1.12 | 3.02 |
| 9.2 | −1.13 | 2.60 |
| 10.6 | −1.14 | 2.51 |
| 11.0 | −1.14 | 2.54 |
| 11.9 | −1.12 | 2.27 |
| 13.0 | −1.09 | 1.72 |

6. The half-wave potential shifted to a more negative potential with increasing pH value up to pH 6, and remained constant at higher alkaline pH values. The reduction waves from pH 4 to 7 ran in two steps, which were most remarkable at pH 5, and the apparent single wave at pH 6 or 7 seemed to be due to an overlap of two waves. Since these two-step waves could hardly be distinguished in the drawing of the polarograms, the values of the half-wave potential in Table I were recorded as a single wave. Because of these phenomena, calculation of shifts in the half-wave potential per pH units seemed to be meaningless.

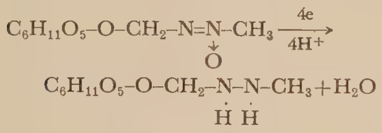
Since cycasin is not stable in alkaline solution, all polarograms at pH higher than 8, were taken twenty-five minutes after the preparation of the electrolytic solution. The limiting currents at pH 1 and 7 had a constant value even after sixty minutes, and the influence of the acid on the limiting current was observed to be reversible. On the contrary, it was observed that the wave height at pH 13 decreased gradually with time.

This reaction in the alkaline medium proceeded irreversibly, that is, after neutralization with acid and adjustment of the solution to a certain acidic pH with buffered solution, the wave height was not recovered to its original value corresponding to the adjusted pH. Thus, it was proved that cycasin is irreversibly decomposed in such a dilute alkaline solution even at low temperature. Therefore, the polarographic behaviors of cycasin were hereafter examined at both pH 1 and 7.

**2. The Limiting Currents at pH 1 and 7** According to the polarographic studies on the aromatic azoxy compounds, previously reported by Levitan et al.[3], Lester et al.[4] and Costa[5], the electrolytic reduction of azoxybenzene follows equation (I):

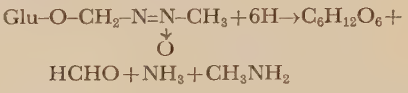

(I)

A similar electrode reaction involving 4 electrons would also be infered for cycasin which has an aliphatic azoxy group in its aglycone. The reduction of cycasin to a hydrazo compound may be represented in the following equation (II). When the number (n) of electrons involved in the electrode reaction is known, the diffusion coefficient (D) of the depolarizer is calculated with the aid of Ilkovič's equation, $I_d=605nCD^{1/2}m^{2/3}t^{1/6}$. From the value of the limiting current obtained at pH 1, and n, shown in equation (II), the value of D was calculated as $4.28\times 10^{-6}$ cm$^2$sec.$^{-1}$ (25°C).


(II)

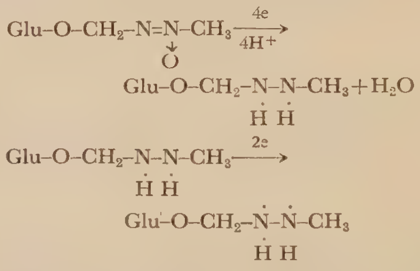
$$C_6H_{11}O_5\text{–O–CH}_2\text{–N=}\underset{\overset{\downarrow}{O}}{N}\text{–CH}_3 \xrightarrow[4H^+]{4e} C_6H_{11}O_5\text{–O–CH}_2\text{–}\underset{H}{\dot{N}}\text{–}\underset{H}{\dot{N}}\text{–CH}_3+H_2O \tag{II}$$

The limiting current at pH 7 was so large that it does not seem to be reasonable to presume that n is equal to 4 in this case. if n was assumed as 6, then, the calculated D should be $4.94\times 10^{-6}$cm$^2$sec.$^{-1}$ (25°C), and it shows fairly good accordance with the above value in the case of pH 1, though the diffusion coefficient is not yet known. It is, therefore, preferable to assume that 6 electrons are involved in the electrode reaction at pH 7. On the other hand, it has been found that in the chemical reduction of cycasin with stannous chloride[2] the reaction proceeds according to equation (III), where Glu is the glucosyl residue.


(III)

$$\text{Glu–O–CH}_2\text{–N=}\underset{\overset{\downarrow}{O}}{N}\text{–CH}_3+6H\rightarrow C_6H_{12}O_6+ HCHO+NH_3+CH_3NH_2 \tag{III}$$

From these facts, the following electrode equation is considered at pH 7 (IV). It is, however, not clearly known how many hydrogen ions do react with the electrode reaction product, since the number of hydrogen ions involved in the electrode process are not known from the $E_{1/2}$ -pH curve.


(IV)

$$\begin{aligned}&\text{Glu–O–CH}_2\text{–N=}\underset{\overset{\downarrow}{O}}{N}\text{–CH}_3 \xrightarrow[4H^+]{4e} \text{Glu–O–CH}_2\text{–}\underset{H}{\dot{N}}\text{–}\underset{H}{\dot{N}}\text{–CH}_3+H_2O\\ &\text{Glu–O–CH}_2\text{–}\underset{H}{\dot{N}}\text{–}\underset{H}{\dot{N}}\text{–CH}_3 \xrightarrow{2e} \text{Glu–O–CH}_2\text{–}\dot{N}\text{–}\underset{H}{\dot{N}}\text{–CH}_3\end{aligned} \tag{IV}$$

For the effect of height (h) of mercury reservior on the limiting current, the results shown in Table II. were obtained. That is, a linear relation exists between the limiting current and the square root of h, and the increase of the wave height at h=60 cm are almost equal to each other. It is, therefore, apparent that these two limiting currents at pH 1 and 7 are both controlled by the diffusion process[6] and do not possess the character of the so-called kinetic currents.[7]

As shown in Fig. 2, the relationships

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[3] N. I. Levitan, I.M. Kolthoff, W.G. Clark and D.J. Tenenberg, *J. Am. Chem. Soc.*, **65**, 2265 (1943).

[4] D. Lester and L.A, Greenberg, *ibid.*, **55**, 496 (1944).

[5] G. Costa, *Ann. triestini univ. Trieste*, Sez. **2**, **2223**, 115 (1953), *Chem. Abst.*, **48**, 4331 (1954).

[6] D. Ilkovič, *Collection Czech, Chem, Soc.*, **6**, 498 (1934).

[7] R. Brdička and K. Wiesner, *ibid.*, **12**, 138 (1947).

TABLE II

DEPENDENCE OF THE LIMITING CURRENTS ON THE HEIGHT (h) OF THE MERCURY RESERVIOR

$2.5 \times 10^{-4}$M Cycasin, at 25°C

| | | pH 1 | | pH 7 | |
|---|---|---|---|---|---|
| h | $\sqrt{h}$ | i | $i/\sqrt{h}$ | i | $i/\sqrt{h}$ |
| 80 cm | 8.94 | 3.20μA | 0.37 | 4.53μA | 0.51 |
| 70 | 8.36 | 3.01 | 0.36 | 4.29 | 0.51 |
| 60 | 7.75 | 2.80 | 0.36 | 4.00 | 0.52 |
| 50 | 7.07 | 2.58 | 0.37 | 3.73 | 0.53 |
| 40 | 6.32 | 2.39 | 0.37 | 3.32 | 0.53 |
| 30 | 5.47 | 2.01 | 0.37 | 2.85 | 0.52 |

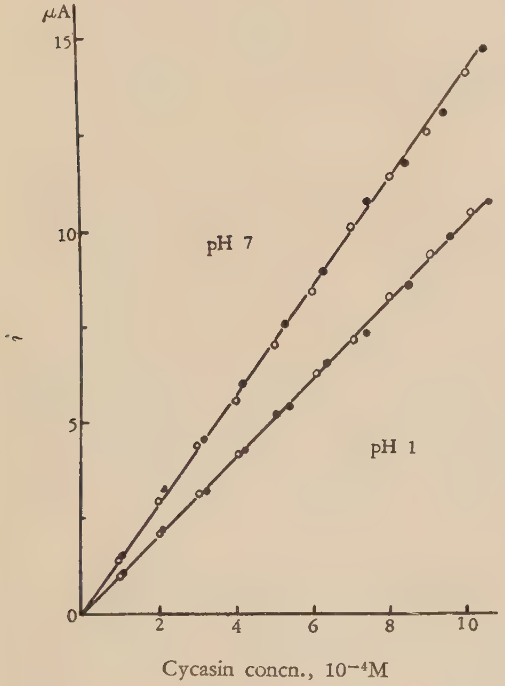

FIG. 2. Relationships between the Limiting Current and the Concentration of Cycasin.

○, solution of cycasin; ●, mixed solution of cycasin, fructose, glucose and sucrose in the ratio of 0.17, 0.04, 0.26 and 1.93.

between the limiting currents and the concentration of cycasin showed linear proportionalities in the range of $10^{-4}$ to $10^{-3}$ M. The equations calculated by the least square method are

$I_d = 1.0230\,C + 0.0513$ at pH 1,
$I_d = 1.4395\,C + 0.0320$ at pH 7,

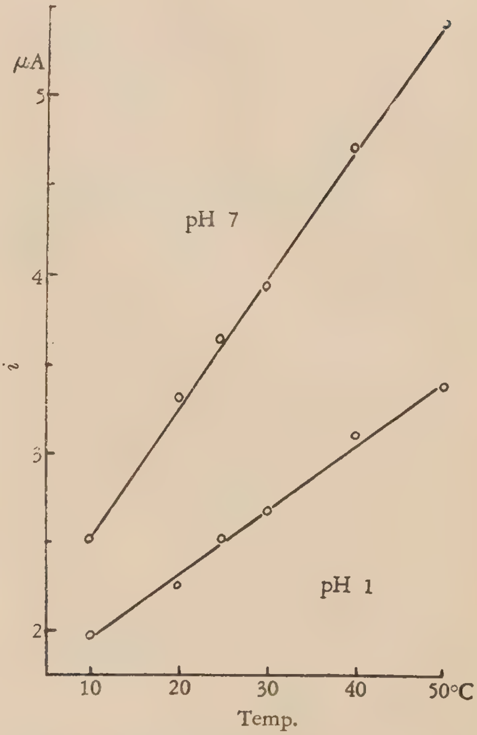

FIG. 3. Influence of Temperature on the Limiting Current of $2.5 \times 10^{-4}$ M Cycasin.

where $I_d$ and C represent the limiting current, μA, and the concentration of cycasin, $\times 10^{-4}$M, respectively.

The results of examination on the effect of temperature on the limiting currents are shown in Fig. 3. The temperature coefficients at 25°C were 1.67 and 2.04% $\mathrm{deg}^{-1}$ at pH 1 and 7, respectively, and were reasonable compared with those found in usual diffusion currents.[8]

**3. Application to the Quantitative Determination of Cycasin** For the purpose of determination of the cycasin contents in cycad seeds, the polarograms of the aqueous alcoholic extracts were inspected. Treatments of the extracts with lead acetate and hydrogen sulfide or potassium oxalate, in the usual manner, proved to be most simple and effective for removal of interfering impurities, such as protein. The coexisting sugars in these extracts were sucrose, glucose and fructose, which were not separated unless chromatographed. As it is indicated in the literature, glucose shows a polarographic wave in alkaline solution only when its concentration is high,9) and fructose also gives a reduction wave whose half-wave potential is more negative.10) The interferences by these sugars would accordingly not appear as a reduction wave itself, but as the influence on the diffusion coefficient of cycasin in the electrolytic solution. For the examination of this point, a mixed solution of cycasin and sugars was prepared according to the ratio found in the matured seeds extracts.* The calibration curves obtained on this mixed solution showed good conformity with those which were obtained when the sugars did not coexist (Fig. 2). It was, therefore, apparent that cycasin can be determined under coexistence of these sugars without any serious interference.

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[8] I. Tachi, "*Polarography*", Iwanami Tokyo, 1954, p. 79.

The extracts used as the sample were the preparations from the matured seeds collected on Oct. 10, 1955, and Feb. 6, 1956, at the suburbs of Nasé City, Kagoshima Prefecture, and were processed as mentioned above. The sample extracts were appropriately diluted and polarographied. The contents of cycasin in the fresh kernels of the seeds, calculated from the wave height, conformed fairly well with the paper chromatographic data, as shown in Table III.

TABLE III
CONTENTS OF CYCASIN IN FRESH KERNEL OF CYCAD SEEDS

| Determined by | | Date of sampling | |
|---|---|---|---|
| | | Oct. 10, 1955 | Feb. 6, 1956 |
| Polarography | at pH 1 | 0.170% | 0.228% |
| | at pH 7 | 0.166 | 0.221 |
| Paper chromatography | | 0.169 | 0.218 |

**5. Conclusion** The direct determination of cycasin can be easily conducted from its polarograms at pH 1 or 7. Being less interfered by impurities, the polarogram at pH 1 gives better results than that at pH 7. The former is also more advantageous because of its smaller temperature coefficient. In paper chromatography, the error in sampling is not so negligible, and procedures such as the development of paper, elution or colorimetry are time consuming and tedious. Polarography supervises in all these points. Being able to separatly determine the coexisting sugars which are difficult to be polarographically determined simultaneously, paper chromatography too, has another superiority. Therefore, these two methods should be preferentially applied, according to the objects.

The authors are gratefel to prof. Dr. Isamu Tachi for his kindness in affording much facilities to carry out this experimental work.

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9) S.M. Cantor and Q.P. Peniston, *J. Am. Chem. Soc.*, **62**, 2113 (1940).

10) p. 357 of 8).

* determined by paper chromatography, cf, ref. 2)

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 3, p. 127~130, 1956]

# Studies on the Flavor of Green Tea

## Part II. Changes in Components of Essential Oil of Tea-leaves

Tei YAMANISHI, Junko TAKAGAKI and Michiyo TSUJIMURA

*Laboratory of Food-chemistry, Ochanomizu University, Tokyo*

Received May 9, 1956

In order to investigate how the flavor of fresh tea-leaves turn into that of green tea, six essential oils were obtained from chopped fresh tea-leaves, whole tea-leaves, and their residues after steaming, green tea and black tea by steam-distillation. They are then compared with each other in regard to their flavor, amount of $I_2$-absorption, $\beta\gamma$-hexenol content and ultraviolet spectrum. It is concluded that the change mentioned above is produced partly by fleeing of $\beta\gamma$-hexenol and partly by some chemical change concerning $\beta\gamma$-hexenol.

There is a considerable difference between the flavor of fresh tea-leaves and green tea.

It has been assumed that this difference was caused by the fleeing of $\beta\gamma$-hexenol from fresh leaves in the process of steaming.[1]

We, therefore, took this subject for our research. First, we found out that when we obtained essential oil from tea-leaves by steam distillation, there was a remarkable difference between the essential oil from the chopped-leaves and that of essential oil from whole-leaves. That is, the aroma of $\beta\gamma$-hexenol (grassy smell) is strong in the former but weak in the latter. Moreover, the color of steaming-residue of chopped-leaves is red-brown like black tea, and its flavor also resembles that of black tea, whereas the residue of whole-leaves is yellowish-green in color and its flavor is very close to that of green tea.

These differences seem to be caused partly by change in the amount of $\beta\gamma$-hexenol and partly by some chemical changes concerning $\beta\gamma$-hexenol.

So we proceeded our study, emphasizing the foregoing point, to find out how the flavor of fresh tea leaves turns into that of green tea.

### EXPERIMENTAL AND RESULTS

### I. Operation of Collecting Essential Oils

**1) Apparatus** We used the apparatus shown in Fig. I.

**2) Operation for obtaining the essential oils.** Fresh tea leaves of the best Sayama tea were used as material for our study.

First, 4 kg of chopped-leaves were put into the boiling boiler which is immediately tightly closed with screw clasps. They were kept steaming till about 3 l of distillate was obtained. This was repeated till 40 kg was treated. The same distillate was obtained from whole-leaves.

The two distillates were saturated with sodium chloride then extracted with an equal volume of ether.

After the ether extracts were dried with sodium sulfate, ether was removed as much as possible by distillating it at 40°C. The crude essential oils thus obtained, are called the first essential oil of chopped-leaves (presented in this paper as C-(1)), and the first essential oil of whole-leaves (W-(1)), respectively.

The steamed residue of chopped-leaves is red-brown in color and its flavor is like black tea, while the steam residue ef whole-leaves is yellowish-green and resembles green tea in flavor.

These residues were stored in an equal volume of ether to avoid putrefaction and about 2 kg of them underwent steam distillation again in the usual way, in a 5 l flask at each time. (Before distillation the residue of whole-leaves was chopped.) The distillates were treated in the same way as in the case of the first essential oils. The essential oils thus obtained, are called the second essential oil of chopped-leaves (C-(2)), and the second essential oil of whole-leaves (W-(2)), respectively.

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[1] S. Takei and Y. Sakoto; *Sci. Pap. I.P.C.R,* (*Japan*) **12**, 13 (1933).

On the other hand, green tea of the best quality and black tea were reduced to powder, there upon they underwent steam distillation for essential oils in the same way as in the case of the second essential oils.

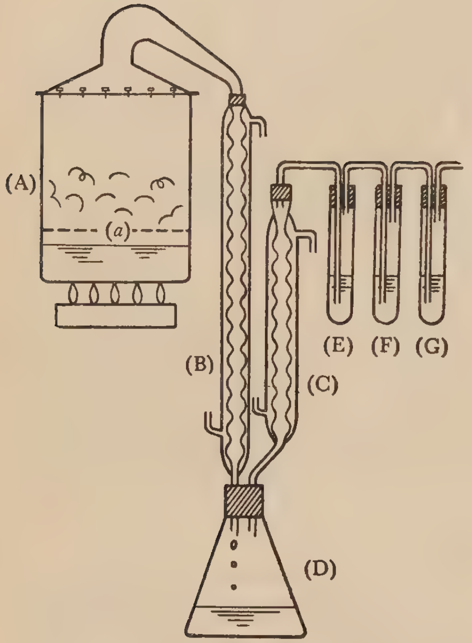


FIG. 1. Apparatus of Steam Distillation.

(A) Boiler: made of aluminum, capacity about 20l, containing about 6l of water below (a)

(B) Allihn's cooler

(C) Allihn's cooler: for catching vapour not condensed by (B)

(D) Large Erlenmeyer's flask: capacity about 3.5l, stoppered by a cork through which (B) and (C) are tightly placed.

(E) Large test tube: contains the mixture of an equal volume of ethanol and ether in order to catch the fleeing vapour.

(F) Large test tube: contains saturated aqueous solution of lead acetate in order to catch hydrogen sulfide and other sulfur compounds.

(G) Large test tube: contains Baryta water in order to catch carbon dioxide.

## II. Comparison of Yield and Nature of These Six Essential Oils

We determined the yields of these six essential oils and examined their nature. (concerning the amount of $I_2$-absorption and flavor). We also determined $\beta\gamma$-hexenol in C-(1) and W-(1), after removal of acids and aldehydes.

The results are given in Table I.

From Table I. the following conclusion may be drawn.

(1) The amount of C-(1) is much larger than W-(1). This difference is chiefly caused by the $\beta\gamma$-hexenol content.

(2) The amount of C-(2) and W-(2) is almost the same, and both are very small in quantity when compared with C-(1) and W-(1). Therefore, the total amount of essential oil from chopped-leaves is much larger than that obtained from whole-leaves.

The following two causes are considered to be attributed to this difference.

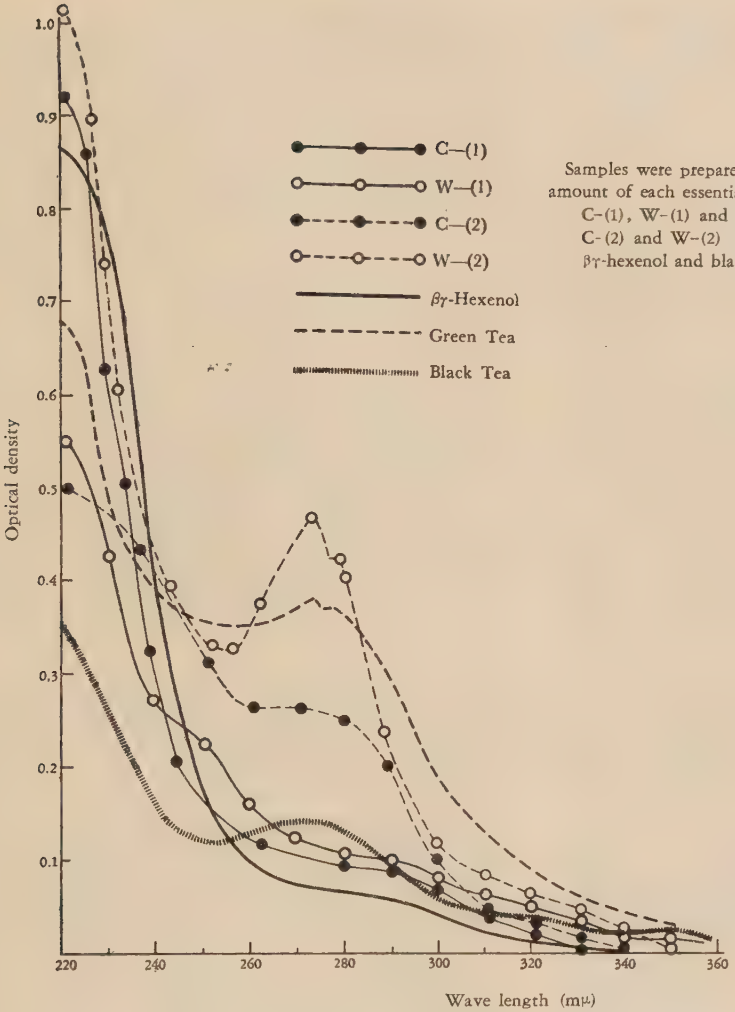
(i) When the fresh tea leaves were chopped, the enzymatic reaction was much activated by exposure to air. Consequently, a large amount of $\beta\gamma$-hexenol was produced.

TABLE I

COMPARISON OF YIELDS AND NATURE OF THE SIX ESSENTIAL OILS

| Essental oil | Yield percent (in dry matter) | Flavor | Amount of $I_2$-absorption (mg per 100 mg of essential oil) | Hexenol content in essential oil |
|---|---|---|---|---|
| C-(1) | 0.0520% | strong grassy smell | 100.8 mg<br>(88.2)* | 35.0% |
| W-(1) | 0.0310 | weak grassy smell and terpen-like aroma | 34.2<br>(46.5)* | 12.0 |
| C-(2) | 0.0020 | black tea-like aroma | 40.2 | — |
| W-(2) | 0.0024 | green tea-like aroma | 47.8 | — |
| Green tea | 0.0138 | aroma of green tea | 31.9 | — |
| Black tea | 0.0383 | aroma of black tea | 80.2 | — |

Note: * data of the previous year.



Samples were prepared by dissolving the following amount of each essential oil in 20ml of pure ethanol.
C-(1), W-(1) and green-tea .......... 3.5mg.
C-(2) and W-(2) .................... 4.2mg.
βγ-hexenol and black-tea ............ 1.75mg.

FIG. 2. Ultraviolet Spectra of Essential Oils.

(ii) If a certain amount of βγ-hexenol is inherently contained in the whole-leaves, some chemical change involving βγ-hexenol will occur in the process of first steaming, and it will turn into some unvolatile substance in whole-leaves.

(3) As for the amount of $I_2$-absorption, C-(1), is about three times as much as W-(1). This difference is considered to be caused by the disparity of the hexenol content according to the report that βγ-hexenol is always contained in essential oil obtained from chopped tea leaves, at a rate of 50~60%.²⁾

## III. Comparison of Ultraviolet Specturm of These Six Essential Oils

We examined the difference in flavor of these six essential oils by determining their ultraviolet absorption spectra. The results are shown in Fig. 2.

Fig. 2. discloses the following points.

(1) The shape of curve of C-(1) resembles that of βγ-hexenol, the concentration of which is one-half of C-(1).

The shape of curve of W-(1) also resembles that of βγ-hexenol, but absorption intensity at 220mμ is much lower than that of βγ-hexenol.

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2) S. Takei and Sakato: *Sci. Pap. I.P.C.R.* (*Japan*). **14**, 1262 (1935).

(2) The shape of the curve of W-(2) differs from that of C-(2). The former shows absorption maximum at 274mμ and 279mμ, in addition to 220mμ, and the shape of its curve resembles that of green-tea. The latter shows no other absorption-maximum than 220mμ, the shape of its curve resembling that of black-tea.

From these results, it is concluded that in the process of first steaming, some substance which shows absorption maximum at 274mμ and 279mμ, is probably produced by some chemical change involving βγ-hexenol and that this newly produced substance gives the characteristic flavor of green tea.

## IV. Test for Components of Fleeing Vapour by Steaming

Fleeing vapour was caught by tubes (E), (F), (G) given in Fig. 1, and tested for aldehydes, sulfur compounds and carbon dioxide.

Aldehydes:

Some aldehydes are detected in the ethanol-ether solution in tube (E) by Tollen's Reagent and Schiff's Reagent, but they were not identified in our experiment.

Sulfur compounds:

A very large amount of hydrogen sulfide was detected by saturated aqueous solution of lead acetate in tube (F) as lead sulfide, but no other sulfur compound could be detected.

Carbon dioxide:

A considerable amount of carbon dioxide was found by Baryta water in tube (G) as barium carbonate. It is considered that a part of fleeing carbon dioxide is in the cells and a part of it is produced by the decarboxylation of certain substances in the process of steam distillation. This subject will be taken up for further study.

## SUMMARY

(1) In obtaining essential oil from fresh tea leaves by steam distillation, chopped-leaves yield a considerably larger amount than whole-leaves, and in concern of the percentage of βγ-hexenol in essential oil, the former is much larger than the latter.

(2) There are many differences in color and flavor of their residues. That is, in the case of chopped-leaves the color is red-brown and the flavor is just like that of black tea, while, in the case of whole leaves the color is yellowish-green and the flavor closely resembles that of green tea.

(3) The essential oils are obtained again from residues previously chopped-leaves and whole-leaves (chopped after the first steaming) by ordinary steam distillation in a 5 l flask. The amount of these essential oils is small, and almost equal in quantity.

The essential oil from chopped-leaves resembles black tea in its flavor and shape of curve in ultraviolet absorption, while in the case of whole-leaves, the flavor and the shape of the curve of ultraviolet absorption resembles green tea.

(4) A considerable amount of hydrogen sulfide and carbon dioxide flee from fresh tea leaves by steam distillation.

The authors wish to express their hearty thanks to Prof. Dr. Ryo Yamamoto of Tokyo Agricultural University and Prof. Dr. Yataro Obata of Hokkaido University for their valuable advice.

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[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 3, p. 131∼134, 1956]

# Studies on the Metabolism of D-Amino Acid in Microorganisms

## Part III. On the Presence of D-Monoamino-dicarboxylic Acid Oxidase

By Shôji MIZUSHIMA and Kin-ichiro SAKAGUCHI

*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*

Received May 10, 1956

In the previous paper it was reported that a mold enzyme preparation from *Aspergillus ustus* strain *f.*, which was found to oxidize D-glutamic acid specifically, was always accompanied by the oxidation of D-aspartic acid. The present study has been carried out to investigate whether or not D-glutamic and D-aspartic acids are oxidized by the same enzyme.

A highly purified enzyme preparation which still shows both activities has been obtained. Several evidences which support the assumption that the both reactions might be catalyzed by a single enzyme, which may be called D-monoamino-dicarboxylic acid oxidase, are also presented.

The dried cells or a crude extract of *Aspergillus ustus* strain *f.*, isolated from soil, were found to oxidize D-glutamic and D-aspartic acids specifically. Many properties of the enzyme responsible for the oxidation of both D-amino acids were reported previously.[1] The purification of this enzyme has now been carried out, and highly purified enzyme preparation has been obtained. Many properties of the enzyme, studied by this purified preparation, are described in this paper.

### EXPERIMENTAL

**Methods and Materials** The enzyme activity is represented by the rate of oxygen uptake in a Warburg manometer at 30°C.

The amount of protein contained in an enzyme preparation was measured by Folin's method which analyses the amount of tyrosine contained in the protein. If an enzyme preparation for example a crude extract, contained non-protein material which was measurable by Folin's method, the amount of these materials was measured by dissolving them in 5% trichloracetic acid, and the value thus obtained was subtracted to give the corrected value for protein material. Succinic acid was determined manometrically using a succinic dehydrogenase preparation from pig heart. Calcium phosphate gel was prepared by the following method:

To 100 ml of 0.44 M $Na_2HPO_4$ was added 100 ml of 0.66 M $CaCl_2$ with vigorous stirring. After adjusting the pH of the mixed solution to 8.2–8.6, the gel was recovered by centrifugation and washed with distilled water until the supernatant fluid showed no Nessler's reaction.

**Purification Procedures of the Enzyme** We obtained 50 g of the dried mycelium of *Aspergillus ustus* strain *f*, from a three days culture grown on 10 litres of medium containing; crude glucose 2%, crude D-glutamic acid 0.5%, $K_2HPO_4$ 0.2% and $MgSO_4 \cdot 7H_2O$ 0.1% in a one to one mixture of distilled water and tap water.

*Step 1. Extraction of the enzyme from dried mycelium.* We ground 10 g of the dried mycelium in a mortar and eluted it with borate-phosphate buffer (pH 8.0) three times through cotton cloth. The eluate was frozen at −20°C overnight, and centrifuged to remove the precipitate. By this procedure 200 ml of a crude extract was obtained.

*Step 2. Treatment with active carbon and protamine.* To the crude extract was added 2 g of active carbon;

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[1] S. Mizushima, K. Izaki, H. Takahashi and K. Sakaguchi This Bulletin, **20**, 36 (1956).

TABLE I
SUMMARY OF PURIFICATION PROCEDURE

| Fraction | Total activity | | Amount of protein | Specific activity µl $Qo_2$/hr/µg. eq. tyrosin | D-A/D-G |
|---|---|---|---|---|---|
| | ml $Qo_2$/hr. | recovery % | µg eq. tyrosin | | |
| Crude extract | 68.5 | 100 | 2300 | 30 | 0.9 |
| After protamine treatment | 59.2 | 87 | 596 | 99 | 0.9 |
| After first dialysis | 46.0 | 67 | 140 | 329 | 0.9 |
| After second fractionation with $(NH_4)_2SO_4$ | 32.8 | 48 | 36 | 911 | 0.9 |
| After first elution from ca-ph-gel | 24.4 | 36 | 20 | 1220 | — |
| After third fractionation with $(NH_4)_2SO_4$ | 14.8 | 22 | 4.9 | 3020 | — |
| After second elution from ca-ph-gel | 10.8 | 16 | 2.7 | 4000 | 1.0 |

this was allowed to stand for 20 minutes with occasional stirring. After centrifugation, 20 ml of 1% protamine solution was added to the supernatant to remove nucleic acids by centrifugation.

*Step. 3. First fractionation with ammonium sulfate.* To the supernatant solution, solid ammonium sulfate was added to raise the concentration to 30% saturation. The precipitate was removed by centrifugation and additional ammonium sulfate was added until a concentration of 60% saturation was reached. The precipitate was recovered by centrifugation, resuspended in 40 ml of the borate-phosphate buffer (pH 8.0) and dialyzed for 4 hours against the same buffer.

*Step 4. Second fractionation with ammonium sulfate.* To the dialyzed sample, solid ammonium sulfate was added to 40% saturation. The precipitate was removed by centrifugation, and, with addition of ammonium sulfate, the supernatant solution was adjusted to 50% saturation. The resultant precipitate was recovered by centrifugation and dissolved in 40 ml of the phosphate buffer (pH 6.0).

*Step. 5. First adsorption and elution of the enzyme from calcium phosphate gel.* We added 20 g of the wet calcium phosphate gel to the enzyme solution, adjusted the pH to 6.0 and mixed for 15 minutes.

The gel precipitate was collected by centrifugation, washed twice with distilled water, and eluted with 20 ml of borate-phosphate buffer (pH 8.0) for 15 minutes. The eluate was obtained by centrifugation. The same eluting procedure with 10 ml of the buffer was repeated twice; thus 40 ml of the eluate was obtained.

*Step 6. Third fractionation with ammonium sulfate.* A fractionation procedure similar to step 4 was carried out, and 20 ml of the enzyme solution was obtained.

*Step 7. Second adsorption and elution of the enzyme from calcium-phosphate gel.*

To the enzyme preparation was added 10 ml of calcium-phosphate gel, the pH was adjusted to 6.0 and this was mixed for 15 minutes. The gel precipitate was collected by centrifugation, washed with distilled water, and eluted using phosphate buffer (pH 7.0) as described in Step 5. In this way 20 ml of purified enzyme preparation was obtained.

The total activity, the specific activity and the activity ratio of the enzyme preparation toward D-glutamic and D-aspartic acids (D-A/D-G) during the enzyme are shown in Table I. At the last step, the purification of the specific activity increased 133-fold. As described later, however, the rate of oxygen uptake of the catalasefree enzyme preparation is twice as large as that of the catalase-containing enzyme preparation. In the initial stages of the purification, the enzyme preparation had a large amount of catalase, whereas the final enzyme preparation had no catalase activity. Therefore, it is reasonable to estimate the increase of specific activity to be about 70-fold. The following experiments were carried out using this final enzyme preparation.

**Specificity** The enzyme preparation showed high specificity toward D-glutamic and D-aspartic acids, and had no activity toward other DL-amino acids such as L-glutamic acid, L-aspartic acid, DL-alanine, DL-methionine, DL-valine, DL-phenylalanine, DL-leucine, DL-tryptophan, DL-threonine, DL-isoleucine and DL-serine.

**Stoichiometric Reaction** As described in the previous report, the crude extract oxidized D-glutamic and D-aspartic acids according to the following equations:

$$\text{D-glutamic acid} + \frac{1}{2}O_2 \longrightarrow \alpha\text{-ketoglutaric acid} + NH_3$$

$$\text{D-aspartic acid} + \frac{1}{2}O_2 \longrightarrow \text{oxalacetic acid} + NH_3$$

However, when the purified enzyme preparation which had no catalase activity was used to examine the chemical balance of the oxidation of D-glutamic acid, the values shown in Table II were obtained. Besides, as hydrogen peroxide was detected iodometrically in the reaction mixture while the reaction was proceeding, hydrogen peroxide was thought to be an initial product in this oxidation.

TABLE II

STOICHIOMETRY OF D-GLUTAMIC ACID OXIDATION BY THE PURIFIED ENZYME

| D-Glutamic acid added | Oxygen consumed | Succinic acid formed | Carbon dioxide formed |
|---|---|---|---|
| 5.0$\mu$M | 4.9$\mu$M | 4.8$\mu$M | 5.4$\mu$M |

Therefore the oxidation of D-glutamic acid, as analyzed with the crude extract and with the purified enzyme preparation, seems to proceed as follows:

Crude extract (containing catalase)

$$\text{D-glutamic acid} + H_2O + O_2 \xrightarrow{\text{oxidase}} \alpha\text{-ketoglutaric acid} + NH_3 + H_2O_2$$

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + \frac{1}{2}O_2$$

$$\text{D-glutamic acid} + \frac{1}{2}O_2 \longrightarrow \text{ketoglutaric acid} + NH_3$$

Purified enzyme (catalase-free)

$$\text{D-glutamic acid} + H_2O + O_2 \xrightarrow{\text{oxidase}} \alpha\text{-ketoglutaric acid} + NH_3 + H_2O_2$$

$$\alpha\text{-ketoglutaric acid} + H_2O_2 \xrightarrow{\text{non enzymatically}} \text{succinic acid} + H_2O + CO_2$$

$$\text{D-glutamic acid} + O_2 \longrightarrow \text{succinic acid} + CO_2 + NH_3$$

When DL-aspartic acid is used as a substrate, oxalacetic acid, the initial product of the oxidation of D-aspartic acid, is easily decarboxylated to give pyruvic acid spontaneously, so that it was impossible to determine the reaction product quantitatively. But even in this case the same reaction mechanism as in D-glutamic acid is thought to exist.

**Michaelis Constant** The Km for D-glutamic and D-aspartic acids has been estimated to be $2.5 \times 10^{-3}$M and $3.2 \times 10^{-3}$M, respectively.

**Effect of Inhibitors** The effect of inhibitors on the rate of oxygen uptake is shown in Table III. Potassium cyanide ($10^{-3}$M) inhibited the enzyme activity almost completely, whereas other metal-chelating inhibitors, such as *αα′*-dipyridil ($10^{-3}$M), sodium fluoride ($10^{-3}$M), ethylenediaminetetraacetic acid ($10^{-3}$M), nitroso-R-salt ($10^{-3}$M), 8-hydroxyquinoline ($10^{-3}$M), and sodium azide ($10^{-2}$M) showed no effect at all. L-Glutamic and L-aspartic acids did not compete with D-aspartic acids for the active site on the enzyme.

TABLE III

EFFECT OF INHIBITORS ON OXYGEN UPTAKE

| Inhibitor \ Substrate | Inhibition (%) | |
|---|---|---|
| | D-Glutamic acid | DL-Aspartic acid |
| KCN $10^{-3}$M | >95 | >95 |
| P.C.M.B. $10^{-3}$M | 53 | 47 |
| Arsenite $10^{-3}$M | 13 | 12 |
| $Ag^{+5} \times 10^{-4}$M | 70 | 80 |

Each vessel contained 20$\mu$M of D-amino acid and a inhibitor in 1.8ml of the borate-phosphate buffer (pH 8.0), 0.2ml of the purified enzyme in the side arm, and 0.2ml of 20% KOH in the centre well. Readings were taken for 20 minutes.

**Rates of Oxygen Uptake for D-Glutamic Acid, DL-Aspartic Acid and D-Glutamic Acid plus DL-Aspartic Acid** Finally, to examine whether D-glutamic acid and D-aspartic acid are activated by a single enzyme the following experiments were carried out. The rate of oxygen uptake was measured under a condition such that the enzyme was saturated with

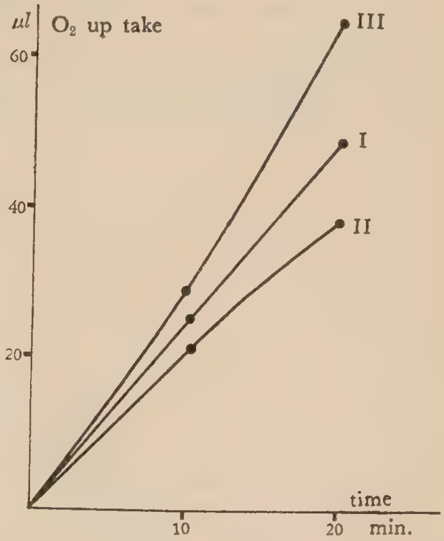

FIG. 1. Oxidation of D-Glutamic and D-Aspartic Acids Saturated for the Enzyme.

Eash vessel contained 0.4ml of the purified enzyme and the borate-phosphate buffer (pH 8.0) in the main compartment, a substrate (1, 20$\mu$M of D-glutamate, 11, 40$\mu$M of DL-aspartate and 111, 20$\mu$M of D-glutamate and 40$\mu$M of DL-aspartate) in the side arm and 0-2ml of 20% KOH in the centre well; final volume 2.2ml.

the substrate; (I) D-glutamic acid, (II) DL-aspartic acid, and (III) D-glutamic acid plus DL-aspartic acid. As shown in Fig. 1, the initial rate of oxygen consumption for each solution of substrate was nearly the same. If D-glutamic and D-aspartic acids were oxidized by different enzymes, the rate for (III) should be the sum of the rates for (I) and (II), even when the enzyme is saturated with the substrate. However the same results as shown in Fig. 1 may also be obtained when D-glutamic oxidase and D-aspartic oxsdase exist separately and D-glutamic acid competes with D-aspartic acid for D-aspartic oxidase, while D-aspartic acid competes with D-glutamic acid for D-glutamic oxidase.

## CONCLUSION

It is natural to consider from following facts that both D-glutamic and D-aspartic acid are oxidized by the same enzyme:

(1) The values D-A/D-G shown in Table 1 remained almost constant throughout the purification process.

(2) The values D-A/D-C of the enzyme preparations obtained from different cultures were also constant.

(3) The optimum pH values for the oxidation of both D-glutamic and D-aspartic acid were found to be 8.0.

(4) The effect of various inhibitors on the rate of oxygen uptake was very similar when either D-aspartic acid or D-glutamic acid was used as a substrate.

(5) As shown in Fig 1, the rate of oxygen uptake for D-glutamic acid plus DL-aspartic acid was not the sum of the rates for D-glutamic acid and DL-aspartic acid alone.

From the facts described above, it may be reasonable to conclude that D-glutamic and D-aspartic acids are oxidized by a single enzyme, D-monoamino-dicarboxylic acid oxidase.

We should like to express our thanks to Mr. Kazuo Izaki for his invaluable advice and assistance throughout this work, and also to Ajinomoto Co. Ltd. for supplying us with D-glutamic acid and other necessary chemicals.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 3, p. 135~138, 1956]

# Studies on Chrysanthemic Acid

## Part V. A Synthesis of *cis*-Caronic Acid

By Masanao MATSUI and Masateru MIYANO

*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*

Received May 14, 1956

cis-Caronic acid (Ia) was prepared by the condensation of methyl $\alpha$, $\beta$-dibromoisovalerate (II) with diethyl malonate followed by hydrolysis and decarboxylation. Ethyl acetacetate or ethyl cyanoacetate was also available in place of malonic ester. Besides *cis*-caronic acid, isopropylidenesuccinic acid (VIII) and isopropenylsuccinic acid (VII) were found in the reaction mixture, however, no *trans*-caronic acid was obtained. This is the first report which submit the preparation of the *cis*-caronic acid with the exclusion of the *trans*-isomer.

*cis*- and *trans*-Caronic acids have been obtained by oxidation or ozonolysis of many terpenes containing $\alpha$,$\alpha$-dimethylcyclopropane ring. Their structural relation to chrysanthemic acids (IIIa), (IIIb) i.e. a possibility to be a useful starting material for a synthesis of natural chrysanthemic acid prompted us to study practical preparation of caronic acid.

Ghosh[1] obtained a mixture of *cis*- and *trans*-caronic acid by the condensation of methyl $\alpha$,$\beta$-dibromoisovalerate with diethyl malonate by methanolic sodium methoxide followed by alkaline hydrolysis and decarboxylation. In our analogous experiments, however the thermal decarboxylotion of the condensed acid (IV), afforded only *cis*-caronic acid (Ia) and no trace of *trans*-modification (racemic modification of (Ib)) could be isolated. isopropenylsuccinic acid (VII), was found also in the thermal decarboxylation products in a comparable amount. The formation of a mixture of *trans*- and *cis*-caronic acid was reported by decarboxylation of the tetracarboxylic acid (V) with dilute hydrochloric acid, and this reaction would, we presume, proceed via the tricarboxylic acid (IV). Treatment of (IV) with 18% hydrochloric acid, however, afforded only terebic acid (VI) along with a small amount of low melting mixed acid.

The condensation of (II) with ethyl acetacetate in methanolic sodium methoxide and subsequent acid decomposition with alcoholic alkali afforded *cis*-caronic acid and isopropylidenesuccinic acid (VIII). Since the formation of the *trans*-acid was also failed in this case, the condensation of the first step must have afforded only *cis*-ketoester (IXa) in place of the expected *trans*-modification (IXb) or their mixture from the stereochemical point of view. This fact gave us suggestion of an stereospecific synthesis of *trans*-caronic acid by oxidizing (X), that is the decarboxylation (ketonic cleavage) product of (IXa). This was not found to be the case, possibly because of the deacylation (acidic cleavage) of (IXa) occurring simultaneously.

The condensation of (II), with ethyl cyanoacetate in a similar manner and subsequent selective hydrolysis of two ester groups and thermal decarboxylation followed by hydrolysis of nitrile resulted again in the formation of only *cis*-acid. This might be due to the formation of intermediate anhydrous acyl imide (XI), which could only exist in *cis*-configuration, as is the case with caronic anhydride.

The most insecticidal natural chrysanthemic

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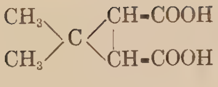
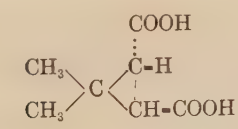
[1] R. Ghosh, *J. Indian Chem. Soc.*, **14**, 449 (1937).


(Ia)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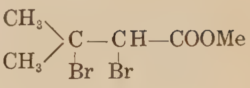
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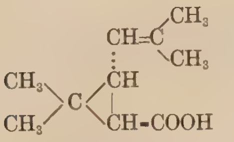
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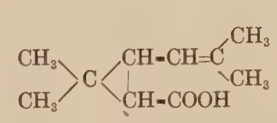
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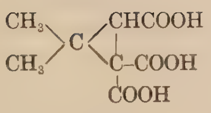
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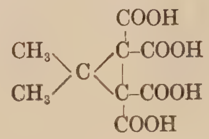
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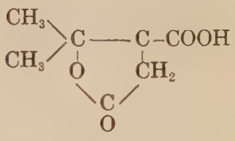
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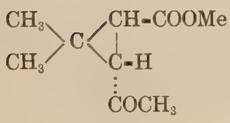
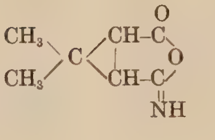
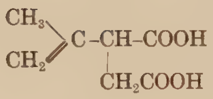
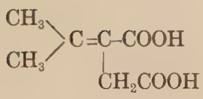
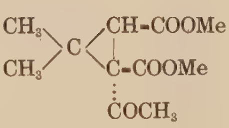
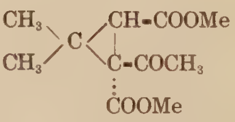

(X)


(XI)


(VII)


(VIII)


(IXa)


(IXb)

acid is the *d-trans*-isomer (IIIa), whose absolute configuration[2] correlates to *l-trans*-caronic acid. In this respect, and further taking into account the troublesome isolation of *trans*-chrysanthemic acid from a synthetic *cis*, *trans* mixture and much more troublesome optical resolution, *trans*-caronic acid was concluded to be the desirable material for our purpose which could be easily resoluted in the *l*-form.[3]

Conversion of *cis*- to *trans*-caronic acid was reported[4] in only a moderate yield. This time, we attempted the conversion of dimethyl *cis*-caronate to *trans*-caronate with base catalysis of sodium methoxide which resulted only in the recovery of the starting material.

The insecticidal activity of *cis*-chrysanthemic acid when converted into allethrolone ester is as much as 70 % of the *trans*-chrysanthemic acid, hence, the synthesis of optical active *cis*-chrysanthemic acid must be an interesting problem in this field. *cis*-Caronic acid having no asymmetric carbon atom is a promissing starting material for this purpose. One of the present authors formerly undertook[5] the synthesis along with this and structural approach to pyrocin.

The authors express their thanks to Professor Y. Sumiki and Dr. R. Yamamoto for their interest in this work. The microanalytical data are indebted to Mrs. K. Satô, Miss M. Suzuki and Miss I. Isobe.

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[2] L. Crombie and S. H. Harper, *J. Chem. Soc.*, **1954**, 470.

[3] This result will be published in subsequent series.

[4] M. Matsui, *Bull. Chem. Soc. Japan*, **23**, 88 (1950).

[5] M. Matsui, *Botyu-Kagaku* **15**, 15 (1950).

## EXPERIMENTAL

**Methyl α, β-Dibromoisovalerate.** Bromine (63 g) in carbon tetrachloride was gradually added to a stirred solution of methyl dimethylacrylate (b.p. 133°, 45 g) in carbon tetrachloride at room temperature. After one hr., the solvent was evaporated and the residue was distilled under reduced pressure. The distillate at 96°/12 mm weighed 104 g, $n_D^{15}$ 1.5080. *Anal.* Found: C, 26.90; H, 4.05; Br, 57.95. Calcd. for $C_6H_{12}O_2Br_2$ requires C, 26.1; H, 4.35; Br, 58.1%.

**Condensation with Ethyl Malonate.** (A) Methyl α, β-dibromoisovalerate (100 g) and diethyl malonate (86.3 g) were dissolved in methanol to which sodium methoxide (from 24.8 g sodium) in methanol (300 ml) was gradually added under stirring in an ice bath, while the temperature rose to ca. 40°C. After the first evolution of heat was subsided, the mixture was heated under reflux for 10 hrs., cooled, then poured into dilute hydrochloric acid containing ice, extracted with ether. The ether-extract was dried with calcium chloride, concentrated and hydrolysed with methanolic sodium hydroxide (80 g of sodium hydroxide was used) for 24 hrs. at 60°. The solvent was evaporated, acidified with hydrochloric acid and extracted with ether (300 ml × 3) and ethyl acetate (300 ml × 3), then the combined organic layer was dried with sodium sulfate. The solvent was removed under reduced pressure and the resulting crystalline mass was heated to 180° for 10 min. The product which mainly consisted of caronic anhydride was dissolved in hot water, treated with active carbon, then crystallized on standing. *cis*-Caronic acid first appeared and from the mother liquor by concentration, and by treatment with active carbon the second crystals were obtained which melted at 146–7° upon recrystallization from water. Both melting point and analytical data are consistent with isopropenylsuccinic acid. *Anal.* Found: C, 53.58; H, 6.14. Calcd. for $C_7H_{10}O_4$ requires: C, 53.16; H, 6.37%. Crude *cis*-caronic acid was recrystallized from hot water, long white needles which melted at 173–4° (decomp.) were obtained. Both melting point and analytical data are consistent with *cis*-caronic acid and depressed upon admixture with terebic acid (m.p. 174°) and isopropenylsuccinic acid obtained above and isopropylidenesuccinic acid (m.p. 156–7°). *Anal.* Found: C, 53.74; H, 6.23. Calcd. for $C_7H_{10}O_4$ requires: C, 53.16; H, 6.37. Although no direct comparison with authentic *cis*-caronic acid was made, the structure was unequivocally assigned by the following reaction together with foregoing evidence. When the acid was treated with boiling hydrobromic acid, an isomeric terebic acid (VII), m.p. 174° (depressed by admixture with the starting acid), was obtained. Secondly, when the acid was distilled, *cis*-caronic anhydride, 154°/15 mm., m.p. 56° (consistent with literature), was obtained in well-nigh quantitative yield, which was analyzed to C, 60.00; H, 5.90. Calcd. for $C_7H_8O_3$ requires: C, 59.99; H, 5.75%.

(B) Methyl α, β-dibromisovalerate (100 g), diethyl malonate (58 g) and sodium methoxide (from 20 g sodium) in anhydrous methanol were condensed and treated as above. The resulting crystalline mass was divided into two before decarboxylation. Eighteen % hydrochloric acid (50 ml) was added and heated under reflux for one hr. Terebic acid (23 g) was crystallized on cooling, m.p. 174–5° undepressed upon admixture with authentic specimen. The other half was heated to 180° for 20 min. and treated in the usual manner. cis-Caronic acid (5 g) and a low melting mixed acid (10 g) which was not further pursued were obtained.

**Condensation with Ethyl Acetacetate.** Sodium methoxide (from 20 g sodium) in 300 ml methanol were added to the cooled solution of methyl α, β-dibromoisovalerate (100 g) and ethyl acetacetate (47 g) in 100 ml methanol. After an initial reaction was subsided, the mixture was heated under reflux for 6 hrs. Sodium hydroxide (60 g) in water (100 ml) was added and refluxed for an additional 18 hrs. The reaction mixture was poured into dilute hydrochloric acid and extracted with ethyl acetate. The extract was dried with sodium sulfate, concentrated in vacuo, cooled and subsequently filtered with suction. The crystals were washed with chloroform, and weighed, 12.5 g, m.p. ca. 145°, when recrystallized from hot water melted at 169–170° which was not depressed upon admixture with *cis*-caronic acid obtained above. The mother liquor was extracted with aqueous sodium bicarbonate solution which was then acidified and evaporated in vacuo. Acetone was added to the concentrate and filtered with suction, and the filtrate was concentrated under reduced pressure. The second crops of *cis*-caronic acid were obtained, 12 g, m.p. ca. 140°, when recrystallized from water it melted at 169–170° not depressed upon admixture with *cis*-caronic acid obtained above and depressed with isopropylidene succinic acid (vide infra) or isopropenylsuccinic acid. The final mother liquor deposited crystals on standing at room temperature which melted at 110–120° consisting of isopropylidenesuccinic acid and *cis*-caronic acid and possibly isopropenylsuccinic acid. From these mixed crystals (10 g) isopropylidenesuccinic acid (teraconic acid) was isolated on recrystallization from water, both m.p. (155–6°, unsharp) and analytical data was consistent with the literature. *Anal.* Found: C, 53.83; H, 6.43. Calcd. for $C_7H_{10}O_4$ requires C, 53.16; H, 6.37%.

**Condensation with Ethyl Cyanoacetate.** Sodium methoxide (from 17.3 g sodium) in methanol (200 ml) was gradually added to the solution of methyl $\alpha, \beta$-dibromoisovalerate and ethyl cyanoacetate in 100 ml methanol under cooling. The mixture was heated at 60° for 12 hrs., cooled and 10 ml water followed by aqueous sodium hydroxide (10 g in 10 ml water) was added and set aside for 24 hrs. Water was added and methanol was removed under reduced pressure and the residue poured into dilute hydrochloric acid containing ice. Ethyl acetate was used for extraction (500 ml × 3). The extract was dried with calcium chloride and concentrated in vacuo, and heated to 180–186° for 12 min. The resulting syrup was hydrolysed with sodium hydroxide (30 g) in water (50 ml) for 6 hrs. under reflux, acidified with hydrochloric acid and extracted with ethyl acetate. After the solvent was evaporated the residue was taken up in hot water, treated with active carbon and crystallized on standing. The crystallization in this case was much slow. *cis*-Caronic acid was obtained, 11 g, m.p. 173–4°.

**Attempted Conversion of Methyl *cis*-Caronate into Methyl *trans*- Caronate.** Methyl *cis*-caronate prepared from *cis*-caronic acid (6.5 g) and diazomethane was heated with sodium methoxide (from one gram sodium) in a small amount of methanol for 10 hrs. on an oil bath (bath temperature ca. 160°). The reaction mixture was treated in the usual manner. The acid melting at 166–7° was obtained, 3.6 g, undepressed on admixture with the starting *cis*-caronic acid. From the mother liquor, the second crystals of *cis*-caronic acid were obtained.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 3, p. 139~140, 1956]

# Calcium Borohydride Reduction of β-Ketoesters

By Masanao MATSUI, Masateru MIYANO and Kiyoshi TOMITA

*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*

Received May 14, 1956

β-Keto esters were smoothly reduced with calcium borohydride into their corresponding hydroxy acids which were identified as α, β-unsaturated acids by dehydration. Tiglic acid, one of these products, was prepared by the other simple process from methyl ethyl ketone in a good yield.

In approaching the synthesis of chrysanthemum dicarboxylic acid, we encountered the reduction of β-ketoester or β-ketonitrile which was much labile and readily sufferable from acidic cleavage (deacylation). Application of sodium borohydride or aluminum amalgum or a restricted amount of lithium aluminum hydride was carried out without success.

van Tammelen et al.¹⁾ prepared the corresponding hydroxy acid by the reduction of methyl 2-methyl-3-carbomethoxy-4-ketoheptadecanoate with sodium borohydride in methanolic solution. Although a fairly good yield (65%) was claimed by these authors, in our preliminary experiment using ethyl acetacetate or ethyl methylacetacetate, we have found that acidic cleavage of β-ketoesters always accompanied, consequently, the yield of hydroxyester was very poor.

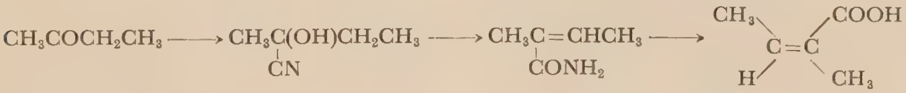
Searching for an appropriate reducing agent which has general applicability in these cases, calcium borohydride was found to be a very favourable one. The borohydride was prepared from commercial sodium borohydride and calcium chloride in cold methanolic solution, which was directly used without isolation or any further purification. The structure of the compound is either (I) or (II) or a mixture of them. Exchange of calcium for sodium was not so rapid and precipitation of produced sodium chloride was complete after 5–30 minutes at 0–10°C.

Crotonic acid from ethyl acetacetate, and tiglic acid (III) from ethyl methylacetacetate were obtained in a moderate yield by reduction with the foregoing reagent followed by dehydration.

Authentic tiglic acid was prepared from methyl ethyl ketone in the following scheme.

$Ca(BH_4)$
(I)

$Ca(BH_4)Cl$
(II)



(III)

Because of its good yield and simple process, this, we presume, is the most practical synthesis of tiglic acid which is of use as vesicant and is specially interesting as a partial structure of chrysanthemum dicarboxylic acid.

The authors express their thanks to Prof. Y. Sumiki and Dr. R. Yamamoto for their interest in this work.

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1) E. van Tammelen, C. E. Osborne, Jr. and S. R. Bach, *J. Am. Chem. Soc.*, **77** 4625 (1955).

## EXPERIMENTAL

**Calcium Borohydride Solution.** Anhydrous calcium chloride (5 g) was added to a cold solution of sodium borohydride (2 g) in methanol (50 ml) and shaken in an ice bath. The end-point of the reaction was indicated by precipitation of sodium chloride.

**Reduction of Ethyl Acetacetate.** Freshly prepared calcium borohydride solution (from 2 g sodium borohydride in 50 ml methanol) was added to a cold methanolic solution of ethyl acetacetate (13 g) under stirring in ice, and the mixture set aside for 2 days at room temperature. Methanol was removed under reduced pressure, hydrochloric acid was added and extracted with ether. The extract was concentrated, hydrolysed with sodium hydroxide in methanol, refluxed for 4 hrs., thereupon the solvent was removed, acidified with hydrochloric acid, then extracted with ether by percolation. The ether extract was concentrated and distilled in the presence of a piece of *p*-toluenesulfonic acid. The distillate (crotonic acid) crystallized readily, the m.p. and mixed m.p. were 72°C, and it weighed 4.5 g (50%).

**Reduction of Ethyl Methylacetacetate.** Freshly prepared calcium borohydride solution (from 2 g of sodium borohydride in 100 ml methanol) was added to a cold methanolic solution of ethyl methylacetacetate (12 g) under stirring in ice and the mixture set aside for 3 days. Methanol was evaporated in vacuo and sodium bisulfate was added to the residue which was then extracted with ether. The extract was concentrated, hydrolyzed with methanolic sodium hydroxide, and the solvent evaporated. Hydrochloric acid was added to the resultant syrup which was then extracted with ether. The ether extract was concentrated and distilled under a somewhat reduced pressure, in the presence of a piece of *p*-toluenesulfonic acid. The half-crystalline distillate was acidified with hydrochloric acid. Ether extraction and evaporation of the solvent afforded tiglic acid as long needles, m.p. 61–2°, mixed m.p. 62–3°, 3 g (37.5%).

**Tiglic Acid.** Crude methyl ethyl ketone cyanhydrin (170 g) prepared in the usual manner was gradually added to a solution of concentrated sulfuric acid (266 g) containing powdered sulfur (2 g) under vigorous stirring in an ice bath while the temperature rose to ca. 50°. The mixture was heated to 150° for 10 min, by an oil bath preheated to 170–180°, then after being rapidly cooled to 50° was poured into ice water, neutralized with sodium hydroxide solution. An additional sodium hydroxide (160 g) was added and it was heated on a steam bath under vigorous stirring for 10 hrs. The cooled mixture was acidified with sulfuric acid and filtered with suction, washed with cold water. Yield 216 g, m.p. 61°, recrystallized from aqueous ethanol or benzene, melted at 63–4°, consistent with literature. The condition selected for isomerization of cyanhydrin was based on the analogous reaction of acetonecyanhydrin.[2]

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[2] Organic Syntheses, Vol. 29, p. 69

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 3, p. 141~147, 1956]

# Sur le Métabolisme Endogène dans la Fermentation Alcoolique de la Levure

par Hiroshi FUKUHARA et Hajimé TAKAHASHI*

*Université de Tokio, Faculté d'Agriculture, Laboratoire de Chimie de Fermentation*

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The endogenous metabolism in the course of glucose fermentation by living yeast cells was studied by means of isotope techniques. The addition of glucose to yeast cells resulted a net acceleration of endogenous $CO_2$ output, and before the cells start the fermentation of added substrate a decomposition of a certain cellular fraction was observed in the presence of 2,4-dinitrophenol. The energetical interpretation of this effect was possible to some extent, but some experimental results do not permit the unitary conclusion.

The source of this endogenous $CO_2$ liberated at the preliminary stage of fermentation was found to be derived from the cold trichloracetic acid-soluble fraction of the cell.

Le métabolisme endogène des microorganismes a été étudié par plusieurs savants[1-5]. Mais les études de cette sorte rencontrent toujours une difficulté essentielle : en présence du substrat extérieur il est impossible de distinguer directement le $CO_2$ endogène de l' " exogène ". A l'aide du carbone radioactif on pourrait déterminer en principe les $CO_2$ provenants des deux sources séparément[6]; mais réellement il se peut bien qu'il existe des réactions d'échange entre les corps intermédiaires du substrat métabolisé et les divers constituants cellulaires.

Les techniques isotopiques, par conséquent, ne peuvent pas toujours donner des résultats corrects. L'emploi du traceur serait, néanmoins, la méthode la plus directe et efficace pour connaître l'état dynamique des cellules vivantes.

Dans ce travail, nous avons examiné avec le traceur C-14 comment le substrat extérieur fait changer le métabolisme endogène de la levure et comment la fermentation du sucre correspond aux comportements des constituants cellulaires, et ce que ces comportements des constituants signifient dans le métabolisme fermentaire de la levure vivante.

## DESCRIPTION DES METHODES

La souche employée est *Saccaromyces cerevisiae.* Le substrat de la fermentation est le glucose dans tous les cas. Le glucose radioactif que nous avons utilisé pour distinguer le métabolisme endogène de l' "exogène" est préparé par la méthode photosynthétique. Le marquage des cellules est pratiqué par la fermentation du glucose radioactif grâce à la levure cultuvée au préalable en aérobiose. Les cellules ainsi marquées sont mises en présence du glucose inactif et le $CO_2$ métabolique est déterminé par manométrie et sa radioactivité est mesurée en même temps par le compteur de Geiger sous forme de $BaCO_3$. Les cellules sont fractionnées d'après la méthode de Schneider[7] modifiée quelque peu. Les fractions sont analysées davantage par les résines à l'échange ionique et par chromatographie sur papier.

La détermination des glucides est faite par la méthode d'anthrone-$H_2SO_4$[8] ou d'après la méthode de Somogyi. Les phosphates sont dosés d'après Allen[9].

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* Université de Tokio, Institut de Microbiologie Appliquée.

[1] J.M. Reiner, H. Gest et M.D. Kamen, *Arch. Biochem.*, **20**, 175 (1949).

[2] H.J. Blumenthal, H. Koffler et E,P. Goldschmidt, *Science*, **116**, 475 (1952).

[3] J.M. Wiame et M. Doudoroff, *J. Bacteriol.*, **62**, 187 (1951).

[4] F.W. Fales, *J. Biol. Chem.*, **193**, 113 (1951).

[5] V. Moses et P.J. Syrett, *J. Bacteriol.*, **170**, 201 (1955).

[6] W.W. Umbreit et al., " Manometric techniques and tissue metabolism ", p. 15 et p. 67 (1951),

[7] W.C. Schneider, *J. Biol. Chem.*, **161**, 293 (1945).

Dans la manométrie du $CO_2$ métabolique radioactif, les fioles de Warburg spéciales sont employées pour absorber quantitativement le gaz radioactif dégagé, après la détermination de la quantité manométrique: avant l'addition du substrat on lave l'intérieur des fioles avec du $CO_2$ inactif puis avec de l'azote pour chasser le gaz radioactif retenu dans le liquide qui contient les cellules marquées. Cette opération est indispensable pour obtenir les résultats reproductibles.

## PARTIE EXPERIMENTALE

**a) Marquage des Cellules.** Quand la levure décompose en aérobiose ou en anaérobiose le glucose radioactif, le C–14 est incorporé dans les cellules à cause de l'assimilation ou des réactions d'échange. La quantité du C–14 incorporé est plus grande dans le cas du marquage aérobique que sous les conditions anaérobiques, mais afin de simplifier les techniques la méthode fermentative est choisie; dans ce cas l'incorporation du C–14 est de 20–25% de la radioactivité totale du glucose ajouté.

La levure est cultivée préalablement sur la machine à secousse à 30°C pendant 24 heures en milieu de culture suivant: glucose 2%, extrait de levure 0.4%, $KH_2PO_4$ 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%. Les cellules sont récoltées, lavées à l'eau distillée, puis épuisées sur la machine à secousse pendant 30–40 heures à 30°C dans une solution de $KH_2PO_4$ à M/100 à pH 4.8–5.2. La levure ainsi obtenue, lavée à l'eau, est mise à incuber sous azote avec du glucose radioactif. Voici un exemple des conditions de marquage:

Un tube de Thunberg contient: suspension des cellules (200 mg à poids sec) 5 ml, tampon de phthalate (M/20, pH 4.6) 5 ml, $KH_2PO_4$ (M/5) 1 ml, solution de glucose radioactif (40–50 $\mu$M) 1 ml. L'incubation est faite à 30°C pendant 2 heures. Le $CO_2$ radioactif dégagé est absorbé dans une solution de KOH.

Dans ces conditions, la décomposition du substrat finit dans les premières 10 à 15 minutes, mais pour fixer la distribution du C–14 dans la cellule on incube encore approximativement pendant 2 heures. L'autofermentation étant beaucoup plus petite que l'autorespiration, la perte de la radioactivité des cellules marquées n'est pas importante pendant cette incubation prolongée. Après l'incubation le tube est refroidi à 2–4°C, puis les cellulles sont lavées à l'eau froide. La distribution du C–14 intracellulaire de la levure marquée sera décrite en détail dans la dernière partie de cet exposé. L'important est que 80–90% de la radioactivité incorporée soit retrouvée dans la fraction acide-soluble.

**b) Fermentation du Glucose par la Levure Marquée.** Les cellules radioactives préparées ainsi contiennent C–14 dans les divers constituants cellulaires, et produisent du $CO_2$ radioactif en présence et en l'absence du substrat extérieur. Par conséquent, quand les cellules décomposent nn substrat ajouté, nous pouvons supposer le changement du métabolisme endogènc au moyen de la détermination de la radioactivité du $CO_2$ dégagé.

La fermentation du glucose par la levure marquée est pratiquée dans le système suivant:

Les fioles de Warbung contiennent: suspension des cellules marquées (dans du tampon de phthalate à M/20; 12 mg de cellules à poids sec, ca. 7700 c.p.m.) 1 ml, solution de $KH_2PO_4$ à M/15 0.5 ml, solution de glucose à M/2 (ou l'eau) 0.2 ml, acide sulfurique pour

TABLEAU I

FERMENTATION PAR LES CELLULES MARQUÉES

| | $CO_2$ dégage ($\mu$l) | Radioactivité du $CO_2$(c.p.m.) |
|---|---|---|
| Glucose ajouté | 600 | 323 |
| Sans substrat | 8 | 115 |

12 mg de cellules à poids sec, 7700 c.p.m. 30 C, sous azote, 15 minutes d'incubation.

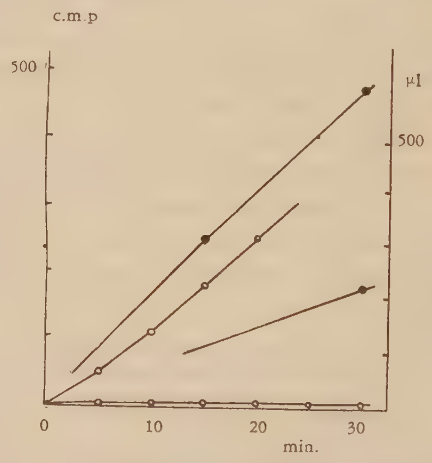

FIG. 1. Fermentation par la levure marquée.

5 mg. de cellules à poids sec; radioactivité totale ca. 5000 c.p.m.; substrat, glucose; ceacles pleins: radioactivité du $CO_2$ dégagé; cercles clairs: manométrie du $CO_2$ dégagé.

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[8] R.J. Allen, *Biochem. J.*, **34**, 858 (1940).

[9] W.E. Trevelyan et J.S. Harrison, *Biochem. J.*, **50**, 299 (1952).

arrêter la réaction 0.3 ml, solution de KOH (4 N, comprend ca. 0.5 M de $CO_3$ entraîneur) pour absorber du $CO_2$ radioactif dégagé 0.5 ml.

La réaction est faite à 30°C, sous azote, pendant 15 minutes.

Le résultat est montré dans le Tableau I. La Fig. 1 montre le dégagement du $CO_2$ radioactif en fonction du temps. En présence de 2,4-dinitrophénol (DNP) ($1.5 \times 10^{-4}$M) une expérience faite sous les conditions semblables (la radioactivité initiale des cellules n'est pas la même) donne le résultat montré dans le Tableau II. Le DNP accélère en général la production endogène du $CO_2$ de la levure en l'absence du substrat extérieur, comme on peut le voir dans le Tableau. En tout cas, la radioactivité du $CO_2$ dégagé est beaucoup plus haute en présence du glucose qu'en son absence.

TABLEAU II

Fermentation par les Cellules Marquées en Présence de 2,4-Dinitrophénol ($1.5 \times 10^{-4}$M)

| | $CO_2$ dégagé ($\mu$l) | Radioactivité du $CO_2$ (c.p.m.) |
|---|---|---|
| Glucose ajouté | 340 | 924 |
| Sans substrat | 32 | 489 |

Un fait semblable a été déjà observé par quelques chercheurs[10].

En utilisant le fait que le DNP possède une action accèlératrice vis-à-vis de la fermentation endogène en l'absence du substrat, nous avons trouvé manométriquement qu'après l'incubation de la levure pendant 2 heures avac le DNP ($1.5 \times 10^{-4}$M) il résultait une dépression presque complète de la fermentation endogène après une production remarquable du $CO_2$ autofermentaire, tandis que l'activité cellulaire dans la fermentation du glucose n'est pas du tout réduite. La fermentation par les cellules radioactives traitées ainsi par le DNP donne le résultat montré dans le Tableau III : le dégagement accéléré du $C^{14}O_2$ par l'addition du substrat est observé ici aussi.

TABLEAU III

Fermentation par les Cellules Radioactives Traitées par 2,4-Dinitrophénol

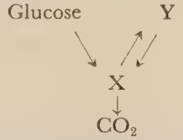
| | Radioactivité du $CO_2$ dégage (c.p.m.) | |
|---|---|---|
| Glucose ajouté | 985 | |
| Sans substrat | 89 | (quantité manométrique est presque zéro.) |

La réaction est faite en présence de dinitrophénol.

**c) Interprétation du Phénomène.** Comme on le voit dans les Tableaux ci-dessus, la radioactivité dégagée par les cellules par l'addition du substrat est très haute et ce fait indique en apparence que l'addition du substrat provoque une fermentation endogène accélérée. Mais nous pouvons-supposer les possibilités suivantes pour expliquer cet effet :

1° S'il existe dans la cellule un grand "pool" des corps intermédiaires de la fermentation du glucose, le "turnover" de ce pool serait très grand pendant la fermentation, et la radioactivité contenue dans ce pool serait diluée de la manière exponentielle par le métabolite qui passe dans la cellule, et le $CO_2$ radioactif serait dégagé au cours de la fermentation du glucose non radioactif.

2° Il existe des réactions d'échange entre les corps intermédiaires de la fermentation du glucose et les constituants cellulaires radioactifs ; c'est à dire dans le schéma, le glucose



est décomposé jusqu'au stade du $CO_2$ à travers le corps X intermédiaire qui peut se transformer réversiblement en Y, constituant cellulaire radioactif, et au cours de la fermentation active le turnover de X grandissant, la radioactivité de la fraction Y apparaitrait dans le $CO_2$ dégagé.

3° La possibilité de l'accélération nette de la fermentation endogène par l'addition du glucose.

La possibilité 1° a été examinée d'abord : si un grand pool des intermédiaires est présent, la radioactivité contenue dans ce pool serait diluée au cours de la fermentation. La quantité du métabolite qui passe dans ce pool étant calculée sur la valeur $Q_{CO_2}$, on pourrait savoir la vitesse de dilution si seulement la grandeur du pool est supposée. Ainsi,d 'après cette calculation les valeurs du Tableau 1 sont trop petites pour qu'il existe un grand pool intermédiaire, or, la forme linéaire de la courbe montrée dans la Fig. 1 n'est expliquée que par l'existence d'un grand pool. Et cette possibilité a été éliminée.

Les possibilités 2° et 3° ne peuvent pas être distinguées directement l'une de l'autre, mais à l'aide du DNP, inhibiteur d'assimilation et de phosphorylation, nous avons examiné indirectement ces deux possibilités.

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10) A. Rothstein et H. Berke, *Arch. Biochem. Biophys.*, **36**, 195 (1952).

Quand la levure décompose en anaérobiose le glucose radioactif en présence du DNP ($1.5\times10^{-4}$M), l'incorporation du C–14 est bloquée environ de 80%. Cela implique que le DNP inhibe l'assimilation et les réactions d'échange en même temps. Par conséquent, si le dégagement du $CO_2$ fortement marqué est observé en présence de DNP pendant la fermentation du glucose par la levure marquée, on en pourrait déduire nécessairement que ce dégagement du $C^{14}O_2$ est dû à la décomposition nette de la certaine fraction cellulaire et non aux réactions d'échange. Le résultat expérimental est rapporté dans le Tableau IV. On y voit un dégagement distinct du C–14 dans le $CO_2$ libéré en présence du substract et de DNP. Cette observation signifie que la fermentation endogène est accélérée par l'addition du substrat.

TABLEAU IV

FERMENTATION DU GLUCOSE EN PRÉSENCE ET EN L'ABSENCE DE DINITROPHÉNOL PAR LA LEVURE RADIOACTIVE TRAITÉE PAR LE DINITROPHÉNOL.

| Système | Radioactivité du $CO^2$ dégagé (c.p.m.) |
|---|---|
| Sans substrat | 36 |
| Glucose | 945 |
| Sans substrat+DNP | 120 |
| Glucose+DNP | 924 |

Concentration finale en DNP est de $1.5\times10^{-4}$M. La viteese de la fermentation à cette concentration en DNP est exactement la même avec la vitesse de la fermentation normale.

**d) Une Expérience sur la Signification Physiologiquede la Fermentation Endogène Accélérée par l'Addition du Substrat.** Comme il a été déjà signalé dans b), les cellules traitées par le DNP montrent une fermentation endogène très dépressée en l'absence du substrat. Quand on ajoute du glucose à ces cellules en présence du DNP, elles ne décomposent le substrat qu'après une phase de latence d'environ 8 minutes, tandis qu'au cours de la fermentation constante qui suit cette phase latente, la vitesse de la formation du $CO_2$ est exactement la même avec la vitesse de la fermentation chez les cellules normales.

Il est déjà admis par beaucoup de chercheurs que le DNP possède une action accélératrice vers l'adénosinetriphosphatase,[11] et cette observation nous fait supposer qu'au commencement de la fermentation par la levure intacte, le cycle énergétique doit travailler activement en fournissant sans cesse de l'énergie, par exemple, de phosphorylation de glucose; la source de cette énergie serait le plus probablement les matières réservées cellulaires qui sous les conditions anaérobiques, se décomposeraient par un mécanisme qui implique la formation du $CO_2$. Par conséquent, lors de l'addition du substrat aux cellules marquées, le dégagement du $CO_2$ à radioactivité spécifique très haute serait observé au stade initial de la fermentation.

Sur la base de cette hypothèse, nous avons poursuivi la radioactivité du $CO_2$ dégagé au début de la fermentation par les cellules marquées: la Fig. 1 montre le résultat. Ici, notre hypothèse ne se réalise pas, la radioactivité spécifique du $CO_2$ n'étant pas particulièrement haute au premier stade. Mais le fait que la radioactivité du $CO_2$ dégagé en l'absence du substrat est aussi considérablement haute (la quantité manométrique étant très faible) nous suggère que le niveau énergétique cellulaire maintenu haut par le métabolisme endogène si actif suffit pour que la levure puisse commencer la fermentation du glucose.

Ainsi nous avons essayé ensuite de découvrir le phénomène supposé, au moyen du traitement par DNP pour abaisser la quantité de l'ATP contenue dans la cellules. Le résultat est montré dans la Fig. 2. On peut voir ici clairement que le dégagement du $CO_2$ radioactif commence au même moment avec l'addition du substrat tandis que l'observation manométrique montre une phase de latence de la production du $CO_2$.

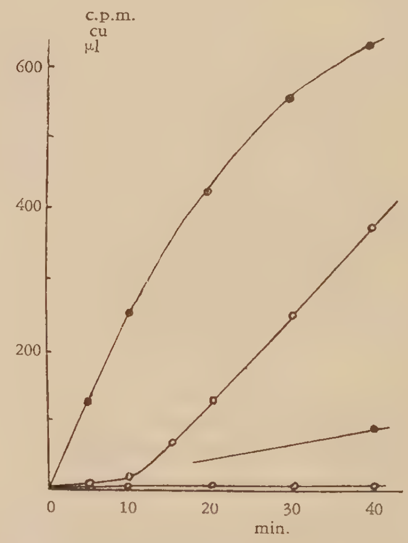

FIG. 2. Fermentation par la Levure Radioactive Traitée par 2,4-Dinitrophénol.

12 mg. de cellules à poids sec; radioactivité totale ca. 7,500 c.p.m.; substrat, glucose; cercles pleins: radioactivité du $CO_2$ dégagé; cercles clairs: manométrie du $CO_2$ dégagé.

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[11] V.R. Potter et R.O. Recknagel, "Phosphorus Metabolism I" p. 382 (1951).

Si le dégagement du $CO_2$ radioactif était dû seulement aux réactions d'échange, les deux courbes, de manométrie et de radioactivité, devraient être parallèles. Par conséquent, au moins dans la levure traitée par le DNP, il est certain que l'addition du substrat provoque une décomposition spécifique d'une certaine fraction cellulaire avant le début de la décomposition du substrat.

Or, si la phase latente causée par le DNP est exclusivement due à la teneur en ATP cellulaire, d'autres poisons du métabolisme énergétique seraient aussi capables de causer ce phénomene; en même temps la teneur en ATP serait basse chez les cellules traitées par DNP. La Fig. 3 et le Tableau V montrent les influences de l'arséniate et la teneur en Δ7 P dans les cellules: l'arséniate est non seulement incapable de causer la phase latente, mais, au contraire, il l'efface, et l'effet inhibiteur de l'arséniate lui-même apparait 20–30 minutes après le début de la fermentation. (Les concentrations variées en phosphate extracellulaire ne montrent aucune influence sur la fermentation.) Et le Δ7 P cellulaire n'est pas réduit par le traitement de DNP.

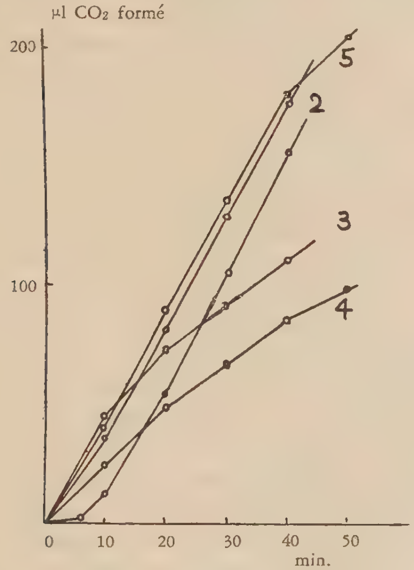

FIG. 3. Effet des Inhibiteurs.

Fermentation sous azote, sans phosphate; tampon de phthalate; 1. contrôle, 2. 2 hrs. d'incubation avec DNP ($1.5\times10^{-4}$M) puis glucose ajouté au temps zéro, 3. Arséniate (M/50), 4. 2 hrs. d'incubation avec arséniate (M/50) puis glucose ajouté au temps zéro, 5. le même système avec 2, arséniate ajouté avec glucose au temps zéro.

TABLEAU V

ANALYSE DES PHOSPHATES DANS LA FRACTION ACIDE-SOLUBLE EXTRAITE DE LA LEVURE

| Condition | P total | P organique | P inorganique | Δ7–P |
|---|---|---|---|---|
| Sans substrat | 1104 (γ) | 513 | 591 | 400 |
| Sans substrat+DNP | 1084 | 513 | 572 | 410 |
| Glucose | 1170 | 677 | 493 | 521 |
| Glucose+DNP | 1143 | 597 | 546 | 445 |

Incubation à 30°C, 30 minutes, sous azote.

Tous ces résultats nous font conclure que la décomposition du constituant cellulaire qui précède la décomposition du glucose ne veut pas nécessairement dire que ce phénomène est la condition obligatoire pour le début de la fermentation au moins dans le sens énergétique.

Quelle que soit la signification de ce phénomène, il s'agit de chercher la source intérieure du $CO_2$ dégagé au premier moment de la fermentation.

**e) Fractionnement des Cellules Marquées.** Les cellules marquées par le processus anaérobique décrit antérieurement sont fractionnées d'abord d'après la méthode de Schneider (l'acide trichloracétique à 10% a été employé.) Le résultat est donné dans le Tableau VI.

TABLEAU VI

FRACTIONNEMENT DES CELLULES MARQUÉES

| Fraction | Immédiatement après la fermentation de glucose radioactif. | Environ 2 hrs. après la fermentation |
|---|---|---|
| ATC froid-soluble | 91% | 85% |
| Alcool-ether-soluble | 1.1 | 1.5 |
| ATC chaud-soluble | 4.6 | 5.9 |
| Résidu | 3.9 | 6.3 |

Conditions de marquage: 200 mg de cellules à poids sec, ca. 50µM de glucose C-14, 30°C, sous azote, 15 min d'incubation.

On voit ici que plus de 80% de la radioactivité incorporée dans la cellule se retrouve dans la fraction acide-soluble, les autres fractions, soit les lipides, les acides nucleiques, et le résidu, n'étant marquées que faiblement. Une distribution semblable a été observée en présence de DNP bien que l'incorporation du C–14 soit beaucoup plus faible dans ce cas.

La distribution du traceur dans les fractions de Schneider est considérablement stable pendant l'incubation prolongée après la consommation complète du glucose radioactif ajouté.

La fraction acide-soluble est ensuite soumise au fractionnement par les résines à l'échange ionique. Les résines utilisées sont Amberlite IR–120 (l'échange des cations) et IRA–410 (l'échange des anions). La

TABLEAU VII
FRACTIONNEMENT DE LA FRACTION ATC-FROID-SOLUBLE PAR RÉSINES

| | | |
|---|---|---|
| ATC froid-soluble | Adsorbé sur IR–120 | 42% (aminoacides) |
| | Adsorbé sur IRA–410 | 32% (acides) |
| | Fraction neutrale | 26% (glucides) |

(exprimé en pour cent de la radioactivité totale incorporée dans la fraction ATC froid-soluble).

fraction acide-soluble est traitée premièrement par IR–120 ensuite par IRA–410. Tous les amphotélytes et les bases sont adsorbés dans IR–120, et les substances acidiques dans IRA–410. Chaque fraction est ensuite analysée par chromatographie sur papier.

Dans la fraction neutrale 73 p. 100 de la radioactivité est contenue dans le tréhalose et 11–20 p. 100 dans le glycérol.

Dans la fraction adsorbée sur IRA–410, à peu près 60 p. 100 de la radioactivité se trouve dans les acides organiques le reste étant distribuée dans les esters phosphorylés.

Dans la fraction adsorbée sur IR–120 toute la radioactivité est comprise dans les acides aminés, particulièrement dans l'alanine (74 p. 100). Nous avons observé que la quantité de l'alanine libre dans la cellule était beaucoup plus grande sous les conditions anaérobiques qu'en présence de l'air, et quant à l'acide glutamique la stuation était inverse.

La distribution du C–14 dans la fraction de résidu est aussi intéressante : cette fraction contient les protéines et les polysaccharides, mais la chromatographie sur papier après l'hydrolyse indique que la radioactivité du résidu se trouve exclusivement dans les hydrolysats des protéines, et les taches du glucose, du mannose ne contiennent pas de radioactivité. Cela signifie que le produit direct de l'assimilation fermentative n'est pas le glycogène.

## CONCLUSIONS ET DISCUSSION

Comme on le sait depuis longtemps, le système zymasique extrait de la levure ne peut pas fermenter le glucose sans l'addition du phosphate d'hexose. L'adénosinetriphosphate était aussi effectif dans nos expériences. Mais chez la levure intacte la fermentation active peut commencer sans aucune phase d'induction. Ce fait nous suggère qu'il existe dans les cellules vivantes un mécanisme énergétique qui induit la fermentation ; cette idée est supportée par l'observation que les cellules traitées par le DNP montrent une phase de latence au commencement de la fermentation et que la décomposition des constituants cellulaires précède la décomposition du glucose. Mais les effets d'autres vingtaine d'inhibiteurs et l'analyse de ⊿7P cellulaire ne nous permettent pas d'en déduire la conclusion unitaire sur l'interprétation du phénomène.

P. Aubert et al.12) ont rapporté en 1955 que dans la fermentation du glucose radioactif la plupart de la radioactivité incorporée dans la cellule s'accumulait dans la fraction acide-soluble cellulaire, particulièrement dans l'alanine, la valine, le tréhalose, etc ; nos expériences semblables sont presque complètement d'accord avec les résultats qu'ils ont acquis. D'après Reiner et al.1), la radioactivité de la fraction acide-soluble est réduite rapidement pendant deux heures d'incubation de la levure en l'absence du substrat et, en place, la radioactivité de la fraction de lipide augmente remarquablement : le $C^{14}O_2$ dégagé au premier stade de la fermentation dérive principalement de cette fraction. Mais dans nos expériences les comportements notables de fraction de lipide n'ont pas été observées.

Par le fait que la radioactivité specifique des composés dans la fraction acide-soluble est très haute et du même ordre avec la radioactivité spécifique du $CO_2$ endogène dégagé au premier moment de la fermentation (à peu près la moitié de la radioactivité spécifique du glucose radioactif employé pour le marquage), nous croyons qu'une certaine substance de la fraction acide-soluble joue un rôle important au commencement de la fermentation chez la levure intacte. Sur ce point il est intéressant de voir que Trevelyan et Harrison13) ont rapporté en 1956 la décomposition du tréhalose au premier stade de la fermentation dans la levure.

Nous sommes heureux de remercier Pro-

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12) J.P. Aubert et G. Millaud, *Compt. rend.*, **240**, 1943 (1955).

13) W.E. Trevelyan et J.S. Harrison, *Biochem. J.*, **62**, 177 (1956).

fesseur K. Sakaguchi et Professeur adjoint B. Maruo qui nous ont donné beauconp de suggestions très utiles.

### RESUME

Le métabolisme endogène de la levure au cours de la fermentation alcoolique a été étudié à l'aide du traceur C-14. La nette accélération de la production endogène du $CO_2$ par l'addition du glucose a été observée en présence de 2:4-dinitrophénol, et nous avons trouvé aussi que la décomposition d'une certaine fraction cellulaire précèdait la fermentation du glucose ajouté.

C'est un phénomène observé pour la première fois sur le metabolisme fermentaire chez la levure vivante. Une interprétation énergétique est possible mais quelques résultats expérimentaux ne permettent pas la conclusion decisive sur ce point.

La fraction acide-soluble cellulaire, qui contient l'alanine, le tréhalose etc, est la source du $CO_2$ radioactif qui se dégage pendant la phase latente qui précède la fermentation du glucose par la levure radioactive traitée par DNP.

[Bull. Agr. Chem. Soc. Japan, Vol. 20, No. 3, p. 148~154, 1956]

# Studies on the Amylolytic System of the Black-koji Molds

## Part I. On the Specificity of Saccharogenic Amylase Fraction

Seinosuke Ueda

*Laboratory of Zymology and Industrial Fermentation, Department of Agriculture,*
*Kyushu University, Fukuoka, Japan*

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Saccharogenic and dextrinogenic amylase fractions were prepared from Black-koji amylase system and their actions investigated with a number of different substrates.

It was found that saccharogenic amylase fraction completely hydrolyzes glutinous rice starch and glycogen to glucose, without leaving any limit dextrin. On the other hand, this enzyme fraction converts potato starch to an extent of about 90% theoretical glucose, the remainder being left as limit dextrin, which is colored purple by iodine. The complete hydrolysis of the branched substrates except potato starch shows that the saccharogenic amylase fraction is capable of hydrolyzing the 1,6-α-D-glucosidic linkage besides the 1,4-linkage, while the branched fraction of potato starch may contain some sort of anomaly to the enzyme. Dextrinogenic amylase fraction hydrolyzes starch and glycogen just as malt α-amylase.

The presence, in mold enzymes, of a "saccharogenic amylase" which seems to be similar to β-amylase of plant origin has been stressed by Tokuoka[1] and Corman et al.[2]. Investigations into its mode of action on starch, and purification of this enzyme have been performed by Kitahara et al.[3], Okazaki[4], Phillips et al.[5], Kerr et al.[6] and Weill et al.[7]. Especially, Phillips et al. suggested that saccharogenic amylase produced by the mold *Rhizopus delemar*, which they referred to as "gluc amylase", splits glucose from the unreducing end-group of starch molecule, and either passes by the anomalous 1,6-α-D-linkage in some manner or else hydrolyzes it very slowly, with difficulty. Recently, this amylase has been crystallized by Fukumoto et al.[8].

In the previous report[9], the author et al. found that the action of Black-koji amylase system on raw starch is much stronger than that of Yellow-koji and malt amylase. In order to clarify the mechanism of raw starch digestion, the action of Black-koji amylase system upon starch paste and glycogen should first be investigated. This paper deals with a study of various preparations and actions of saccharogenic and dextrinogenic amylase fractions upon starch paste of different origin and glycogen.

### MATERIALS AND EXPERIMENTAL

**Enzyme preparations.** Saccharogenic Amylase Fraction: This amylase is less sensitive to exposure than the accompanying dextrinogenic amylase of its aqueous solution, to high hydrogen ion concentrations at a relatively high temperature. This property made possible the removal of dextrinogenic amylase from this Black-koji amylase system. The wheat bran, inoculated with strain Kawachi of *Aspergillus awamori*, incubated at 30°C for 4 days, was extracted for about 5 hours with 10-folds of tap water in the presence of toluene. The filtrate was dialyzed over-night against tap water under toluene, and then brought up to pH 2.4 with 1 N HCl. The solution, heated to an extent of 60°C for 15 minutes at pH 2.4, sufficient enough to destroy dextrinogenic amylase activity selectively, was then neutralized with 1 N sodium citrate to pH 3.8 and cooled down to room temperature. After filtration, a clear light brown solution was concentrated in vacuum at 38°C to the range of one-tenth to one-twentieth of the initial volume. This concentrated solution was again dialyzed over-night against tap water, and used directly as the enzyme solution of the saccharogenic amylase fraction. This fraction also possessed considerable maltase activity.

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[1] Y. Tokuoka, *J. Brew. Soc. Japan*, **19**, 791 (1941).

[2] H. J. Corman and A.F. Langlykke, *Cereal Chem.*, **25**, 191 (1948).

[3] K. Kitahara and M. Kurushima, *J. Ferm. Tech. Japan*, **27**, 1, 44, 182, 213, 254 (1949), **28**, 106, 388, 422 (1950).

[4] A. Okazaki, *J. Agr. Chem. Soc. Japan*, **24**, 88, 201 (1950), **25**, 224 (1951), **25**, 317, 321 (1952), **26**, 447 (1952), **28**, 48, 51 (1954), **29**, 181, 273, 277, 605 (1955).

[5] L. L. Phillips and M. L. Caldwell, *J. Am. Chem. Soc.*, **73**, 3559, 3563 (1951).

[6] R. W. Kerr. F. C. Cleveland and W, J. Katzbeck, *J. Am. Chem. Soc.*, **73**, 3916 (1951).

[7] C. E. Weill and R.J. Burch. *Cereal. Chem.*, **31**, 150 (1954).

[8] J. Fukumoto, Y. Tuzizaka and K. Minamii, *Symposia on Enzyme Chem. (Japan)*, **9**, 94 (1954).

[9] I, Yamasaki and S. Ueda, *J. Agr. Chem. Soc. Japan*, **24**, 181 (1951).

Dextrinogenic Amylase Fraction: *Aspergillus usamii*, one of the Black-koji molds, was grown in shaking culture, inoculating with spores, in the following special medium for 48 hours at 24°C. It was filtered and the filtrate concentrated in a reduced pressure at 38°C, to the range of one-tenth to one-twentieth of its initial volume, then dialyzed overnight under toluene in a collodion sack against tap water. This dialyzed enzyme solution was used directly as the dextrinogenic amylase fraction, though maltase activity still remained.

A culture medium; Raw corn starch, sterilized by dry heat at 160°C for 1 hour, 1%, urea, 0.2%, $MgSO_4 7H_2O$ 0.1%, NaCl 0.02%, $KH_2PO_4$ 0.2% and glucose 0.1%.

This culture medium allowed the mold to grow only very slowly and hence, enabled us to distinguish the preceding period of growth of about 48 hours, in which the pH changed gradually from 6.4 to 5.2 and dextrinogenic amylase plus maltase were secreted preferentially, from the following main period of growth of about 24 hours in which the pH dropped rapidly from 5.2 to 3.0 and secretion of saccharogenic amylase began.

A strain of *Aspergillus awamori*, the most popular "Black-koji" mold, which was mostly used in our work, was found unable to grow in this medium because of the poor utilization of urea by the strain.

β-Amylase of Barley: It was prepared from ungerminated barley after the method of Blom et al.[10]. This preparation showed neither dextrinogenic activity nor maltase activity.

**Preparations of Substrates.** Preparation of β-Limit Dextrin (β-LD): β-LD was prepared from potato starch, soluble starch (potato) and glycogen, all of which were obtained from commercial sources, by β-amylase of barley described above in accordance with the usual method.

Preparations of Amylopectin and Amylose from Potato Starch: Amylopectin was prepared after the method of Haworth et al.[11], and purified after the method of Krishnaswamy et al.[12]. Besides, it is notable that amylose and amylopectin prepared from potato starch by Schoch's method, were kindly supplied by Prof. Nikuni.

Preparation of Glutinous Rice Starch: This starch was prepared in the usual manner in this laboratory.

**Determination of Enzyme Activity.** Dextrinogenic Activity: Five ml of enzyme solution was added at 50°C, to a mixture of 25 ml of 0.8% boiled soluble starch solution, 5 ml of 0.1M citrate buffer at pH 3.8 and 5 ml of distilled water (total volume 40 ml). While the reaction mixture was kept at 50°C, then at suitable time-intervals (usually 1 to 2 minutes), two portions (0.5 and 1.0 ml) were taken out from the reaction mixture. One portion (0.5 ml) was used in the observation of the color change by adding 5 ml of one-thousandth normal iodine solution and hence in determining the time elapsed before the achromatic stage was reached. The other (1.0 ml) was used in determining the reducing sugar formed by the micro-Bertrand method[13] and hence in calculating the degree of hydrolysis in terms of per cent of glucose formed on the theoretical glucose value of starch. For convenience sake of comparison, one unit of dextrinogenic activity was defined as that which is able to convert starch to the achromatic point with iodine in 25 minutes under the above conditions.

Saccharogenic Activity: Composition of the reaction mixture was the same as above. Five ml of saccharogenic amylase fraction was used as enzyme solution. After the reactions in 5, 10 and 30 minutes respectively, at 50°C, the reducing sugar formed was determined by the micro-Bertrand method and the degree of hydrolysis was calculated as described above. One unit of saccharogenic amylase activity was defined as that which converts 1% of starch in the reaction mixture to glucose per minute under the above conditions.

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[10] J. Blom, A. Bak and B. Braae, *Z. physiol. Chem.*, **241**, 273 (1936).

[11] W. N. Haworth, S. Peat and P.E. Sagrott, *Nature*, **157**, 16 (1946).

[12] K. G. Krishnaswamy, *J. Biol. Chem.*, **176**, 1253 (1948).

[13] G. Klein, Handbuch der Pflanzenanalyse II, Spezielle Analyse I, 786 Wien (1932).

Maltase Activity: Composition of the reaction mixture was also the same as above except for the substrate. A 0.8% maltose solution was used as the substrate instead of starch solution. After each 0, 5, 10 and 30 minutes of reaction at 50°C, the reducing sugar formed with maltose remaining, was determined and the degree of conversion of maltose calculated in per cent. One unit of maltase activity was defined as that which converts 1% of maltose to glucose per minute under these conditions.

**Hydrolysis of Various Starches and Glycogen.** Starches obtained from various sources and glycogen were hydrolyzed under the following conditions, which had by preliminary experiments been found optimum in respect of stability and activity of the enzymes. Fifty ml of 1% cooked starch or glycogen solution, 10 ml of 0.1 M citrate buffer at pH 3.8, 10 ml of enzyme solution and 30 ml of distilled water were mixed together at 50°C making the total volume 100 ml and incubated at 50°C for 360 minutes. Whenever some extra amount of any kind of enzyme solution was added, the same amount of distilled water was reduced correspondingly. Aliquots were removed from hydrolysate at suitable time-intervals, in order to measure the reducing sugar and the color change with iodine.

**Paper Chromatography.** Droplets (ca. 0.02 ml) of the hydrolysate were repeatedly applied to a strip of Toyo filter paper (No. 5 B), 5 cm-width and 40 cm-length at a distance of 3 cm from one end and 1.5 cm from one side. Concentrated Saké, as the control, was applied at a distance of 1.5 cm from the other side, because Saké contains glucose, isomaltose, panose and so on, as reported by Aso et al.[14]. After drying, the end of the strip was immersed in a solvent mixture consisting of butanol (6 parts), pyridine (4 parts) and water (3 parts), and the strip was suspended for 16 hours in a closed chamber at room temperature, which was afterwards removed to air-dry. This procedure was repeated four times. After spraying with a solution prepared by adding aniline (0.39 g) and phthalic acid (1.66 g) to water-saturated butanol (100 ml), the strip was heated in an air oven at 120°C for 15 minutes. The position of the red-brown colored spots on the paper strip was then determined.

## RESULTS

Activity of Dextrinogenic Amylase Fraction: The enzyme solution contained no trace of $\alpha$-amylase action as confirmed by Wijiman's method. And when acted on starch paste, it showed a digestion curve which changed sharply at about 20 to 30% hydrolysis. The iodine reaction of starch disappeared a little before the digestion curve reached a break. These properties are quite similar to the dextrinogenic amylase of malt. It was, therefore, denoted as the dextrinogenic amylase fraction.

Dextrinogenic activity, denoted in time-length up to the achromatic stage, was directly proportional to the enzyme concentration as shown in Fig. 1.

Activity of Saccharogenic Amylase Fraction: Since this enzyme solution formed reducing sugar without disappearance of the characteristic iodine reaction during the whole course of digestion of soluble starch, it was designated as the saccharogenic amylase fraction.

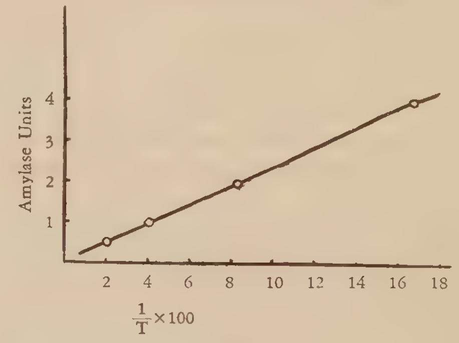

FIG. 1. The Relationship between Dextrinogenic Amylase Units and the Time, Required to Reach the Achromatic Stage.

Comparison of Activity of Various Enzyme Solutions: Dextrinogenic, saccharogenic and maltasic activities of the various preparations, represented in units, are shown in Table I.

Extent of Hydrolysis of Starches and

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[14] K. Aso, K. Shibazaki and F. Yamauchi, *J. Ferm. Tech. Japan*, **30**, 311 (1952), **32**, 47 (1954).

TABLE I

Comparison of Activities of various Enzyme Solutions

| Designation | Source of preparation | Dextrinogenic Activities | Saccharogenic Activities units | Maltasic Activities |
|---|---|---|---|---|
| Dextrinogenic amylase fraction I | Shaking culture of mold | 1.25 | (0.90) | 0.50 |
| Saccharogenic amylase fraction I | Mold bran | 0 | 3.98 | 2.53 |
| Saccharogenic amylase fraction II | Mold bran | 0 | 1.45 | 0.90 |
| β-Amylase | Barley | 0 | 1.60 | 0 |

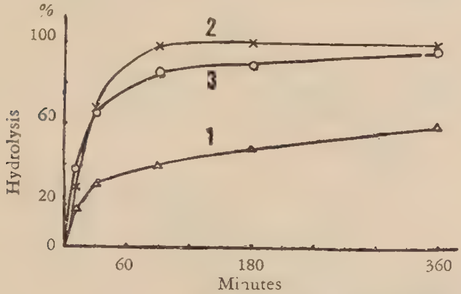

FIG. 2. Hydrolysis of Glutinous Rice Starch by Two Fractions of Black-Koji Amylase System.

Curve 1, dextrinogenic amylase fraction-1 alone; Curve 2, saccharogenic amylase fraction-1 alone; Curve 3, dextrinogenic plus saccharogenic amylase fraction;

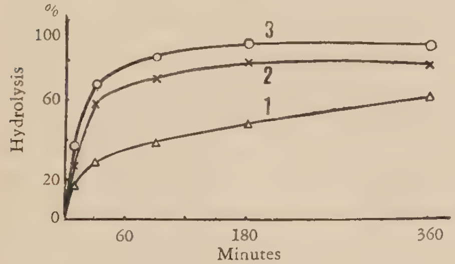

FIG. 3. Hydrolysis of Potato Starch by Two Fractions of Black-Koji Amylase System.

Curve 1, dextrinogenic amylase fraction-1 alone; Curve 2, saccharogenic amylase fraction-I alone; Curve 3, dextrinogenic plus saccharogenic amylase fraction;

Glycogen. Using dextrinogenic amylase fraction alone (curve 1 in figures 2, 3 and 4), various kinds of cooked starches, that is, glutinous rice starch, potato starch and soluble starch (potato), were equally converted to an extent of 60 to 64% in 360 minutes at 50°C, each showing a distinct break at about 30% hydrolysis in the digestion curve shortly after the extinction of iodine reaction. Slow increase from 30 to 60% hydrolysis would be due to the weak saccharifying action of dextrinogenic amylase fraction on the dextrins formed. While, in the case of glycogen (curve 1 in Fig. 5) the extent of hydrolysis was merely 15% and hardly proceeded after 60 minutes. These features are quite similar to that reported on malt α-amylase by Myrbäck.15)

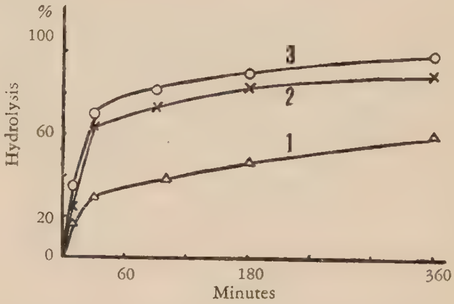

FIG. 4. Hydrolysis of Soluble Potato Starch by Two Fractions of Black-Koji Amylase System.

Curve 1, dextrinogenic amylase fraction-1 alone; Curve 2, saccharogenic amylase fraction-1 alone; Curve 3, dextrinogenic plus saccharogenic amylase fraction;

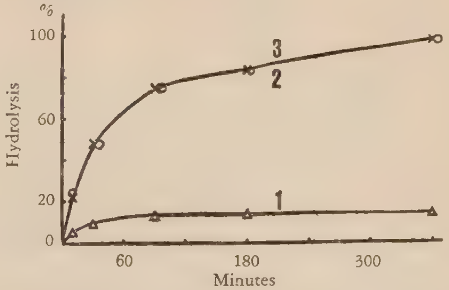

FIG. 5. Hydrolysis of Glycogen by Two Fractions of Black-Koji Amylase System.

Curve 1, dextrinogenic amylase fraction-1 alone; Curve 2, saccharogenic amylase fraction-1 alone; Curve 3, dextrinogenic plus saccharogenic amylase fraction;

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15) K. Myrbäck, Advancesi n Carbohydrate Chem., **3**, 251 (1948).

On the other hand, by using saccharogenic amylase fraction (curve 2 in figures 2,3,4 and 5), glutinous rice starch and glycogen were converted completely up to 100% in 360 minutes at 50°C. The digestion of glutinous rice starch, especially proceeded rapidly and the 95% hydrolysis was completed in 90 minutes, while in the same length of time about 75% of glycogen was hydrolyzed. But the extent of potato starch hydrolysis was 90 to 92%, that of soluble starch 80%, each showing a break at about 70% hydrolysis in the conversion curve. The iodine reaction always remained purple until the very end of reaction in 360 minutes.

By mixing both fractions of the enzyme (curve 3, in figures 2, 3, 4 and 5), potato starch was completely converted up to 100% in 360 minutes at 50°C, while soluble starch was unable to be converted over 90% with a sort of limit dextrin which was left in the hydrolysis. The nature of this dextrin will be worth to investigate.

Extent of Hydrolysis of Glutinous Rice Starch: Glutinous rice starch, previously converted up to the final extent of 49% as maltose by barley β-amylase, also, was rapidly hydrolyzed completely up to 100% by saccharogenic amylase fraction alone, which was added after destroying the barley β-amylase by heating (Fig. 6).

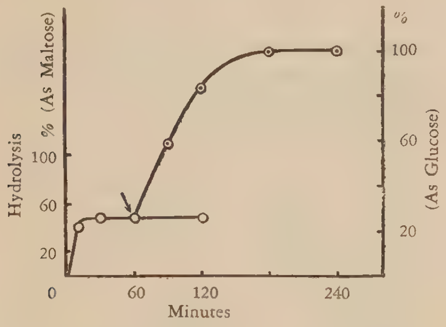

FIG. 6. Hydrolysis of Glutinous Rice Starch by Barley β-Amylase, Afterwards by Saccharogenic Amylase Fraction-I.

Arrow indicates the addition of saccharogenic amylase fraction.

In one of the experiments wherein the hydrolysis of glutinous rice starch was carried out to completion, the hydrolysate was analyzed by paper chromatography. The sugar produced in the hydrolysate was found to be glucose only, no other reducing sugars being found in a detectable amount. Furthermore, the hydrolysate, after its fermentable sugar was removed with bakers' yeast, was concentrated to one-tenth of the initial volume and then analyzed by paper chromatography once more. In this case also, no other reducing sugars except glucose were detected.

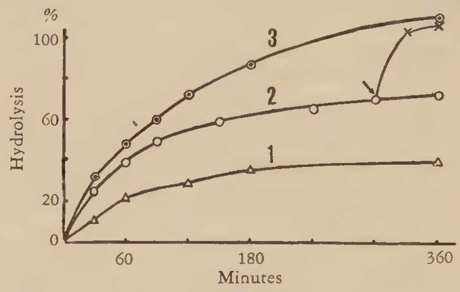

FIG. 7. Hydrolysis of β-Limit Dextrin by Saccharogenic Amylase Fraction-I.

β-Limit dextrin from soluble starch (—△—△—); that from potato starch (—○—○—); that from glycogen (—◎—◎—); Arrow indicates the addition of dextrinogenic amylase fraction-I.

Extent of Hydrolysis of β-LD and Amylopection: The results were given in figures 7 and 8. β-LD prepared from glycogen (curve 3 in Fig. 7) was smoothly and completely converted to glucose by the saccharogenic amylase fraction alone. Whereas, β-LD from potato starch (curve 2 in Fig. 7) was hydrolyzed only to an extent of 70% in 360 minutes, and the hydrolysis curve showed a dull break at about 50%. But if the dextrinogenic amylase fraction was added thereto (at the arrow point on curve 2), hydrolysis was promptly accelerated and rapidly reached the final extent of about 100%. β-LD from soluble starch (curve 1 in Fig. 7) showed peculiarity of being saccharified only to an extent of 40% and not more, by the saccharo-

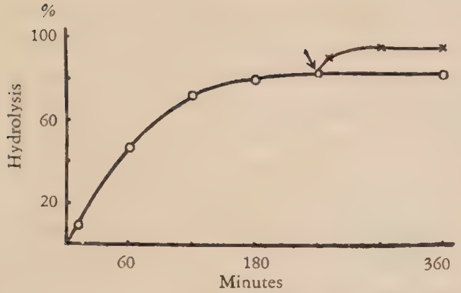

FIG. 8. Hydrolysis of Amylopectin of Potato Starch by Saccharogenic Amylase Fraction-II and Effect of Addition of Dextrinogenic Amylase Fraction-I thereto.

genic amylase fraction.

Amylopectin from potato starch gave the hydrolysis curve as illustrated in Fig. 8. With the saccharogenic amylase fraction alone, the curve showed a sharp change at about 70 % hydrolysis and ultimately reached an extent of 85 %, whereas, as a result of this, a sort of limit dextrin which was colored purple by iodine was left. But at this point (at the arrow point) when the dextrinogenic amylase fraction was added thereto, the chromogenic property with iodine disappeared on the spot and the saccharification was restarted, soon reaching an extent of about 97 % hydrolysis. Here, the above result also revealed the supplementary role of dextrinogenic amylase fraction to saccharogenic amylase fraction in complete hydrolysis of potato starch.

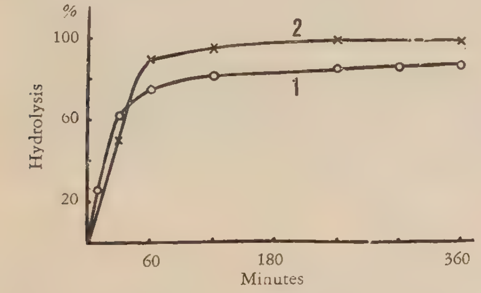

FIG. 9. Hydrolysis of Amylopectin and Amylose by Saccharogenic Amylase Fraction-I.

Curve 1, amylopectin; Curve 2, amylose;

Extent of Hydrolysis of Amylose* and Amylopectin* Prepared from Potato Starch: As shown in Fig. 9, using the saccharogenic amylase fraction alone, amylose was hydrolyzed up 100 % but amylopectin up to 88%, and a limit dextrin colored purple with iodine remained.

Effect of Adsorption of Saccharogenic Amylase Fraction on Corn Starch: Corn starch powder was stirred into the cold saccharogenic amylase fraction (20 g per 100 ml) at pH 3.6 for 30 minutes, and then removed by centrifugation. The activities of the treated solution are shown in Table II. Using these two enzyme solutions, β-LD from glycogen and from potato starch were hydrolyzed comparatively (Fig. 10). As shown in Fig. 10, it is a remarkable fact that, because of adsorption treatment, saccharogenic amylase fraction lost most of its saccharifying

TABLE II

EFFECT OF ADSORPTION OF SACCHAROGENIC AMYLASE FRACTION ON CORN STARCH

| | Saccharogenic units | Ratio | Maltasic units | Ratio |
|---|---|---|---|---|
| Activities before treatment | 3.50 | 100 | 2.53 | 100 |
| Activities after treatment | 1.80 | 51 | 1.42 | 56 |

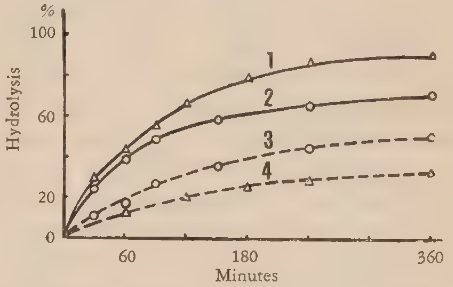

FIG. 10. Effect of Adsorption of Saccharogenic Amylase Fraction with Corn Starch on the Hydrolysis of β-Limit Dextrin.

The continuous curves represent saccharogenic amylase fraction without treatment, whereas the broken curves represent treated saccharogenic amylase fraction.

Curve 1, and 4, β-limit dextrin from glycogen (—△—), Curve 2 and 3, β-limit dextrin from potato starch (—○—).

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* Preparations from Prof. Nikuni.

activity towards β-LD from glycogen, but not so towards that from potato starch.

## DISCUSSION

Curve 2, in Figs. 2, 3, 4 and 5, respectively shows the course of hydrolysis carrid out by the saccharogenic amylase fraction on various kinds of amylous materials. From such results attained in these experiments as the hydrolysis of the branched fraction of potato starch was 88 % theoretical glucose, of glutinous rice starch 100 %, of glycogen 100 %, it is suggested that saccharogenic amylase fraction either hydrolyzes 1,6-α-D-glucosidic linkage as easily as 1,4-linkage or passes by the linkage in some manner, as Phillips et al. said.[5] However, the latter suggestion does not seem to be plausible in this case, because the failure of saccharogenic amylase fraction to hydrolyze isomaltose and dextran was settled in the preliminary experiments. Moreover, after treatment of corn starch adsorption, the effluent of the amylase fraction hydrolyzes β-LD from glycogen more difficultly than that from potato starch, just in opposition to the initial amylase fraction. From this fact it may be concluded that saccharogenic amylase fraction contains a "debranching factor" which is more easily adsorbable onto corn starch than saccharogenic amylase itself. But it must be investigated further whether this factor represents isoamylase,[16] amylo-1,6-glucosidase,[17] or a sort of amylase with debranching activity. Potato starch is converted by saccharogenic amylase fraction to an extent of about 90 % and remains dextrin stained purple with iodine. It is suggested, therefore, that potato starch has an anomalous linkage besides the 1,6-linkage, which arrests the amylolysis by saccharogenic amylase fraction. The action of dextrinogenic amylase fraction on amylous materials appears to be quite similar in a general way to malt α-amylase, by which potato starch and glutinous rice starch are attacked more easily than glycogen and in the course of their hydrolysis, a sharp change occurs on the hydrolysis curve at the level of about 20–30 % conversion. The reason why glutinous rice starch is saccharified much easier than glycogen must be claimed due to the higher degree of branching for glycogen.

## SUMMARY

Using preparations of dextrinogenic and saccharogenic amylase fractions of Black-koji amylase system, the conversion of starches and glycogen was investigated.

1) The mode of conversion of amylous materials by dextrinogenic amylase fraction seems to be the same with that of malt α-amylase.

2) Saccharogenic amylase fraction hydrolyzes the glutinous rice starch and glycogen completely to glucose in spite of the existence of 1,6-linkage leaving no other sugar detectable by paper chromatography.

3) The adsorption treatment of this saccharogenic amylase fraction with corn starch makes the hydrolysis of the β-LD from glycogen more difficult than that from potato starch just in opposition to the case with the initial amylase fraction. It may, therefore, be considered here that some "debranching factor" exists in this amylase fraction.

4) Saccharogenic amylase fraction hydrolyzes potato starch to an extent of 90 % leaving a sort of dextrin colored purple by iodine. Hence, it is suggested that potato starch has an anomalous linkage to the enzyme.

The author wishes to acknowledge his indebtedness to Prof. Yamasaki for his valuable advice and criticism. The author also wishes to thank Prof. Nikuni who kindly furnished amylose and amylopectin.

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[16] B. Maruo and T. Kobayashi, *J. Agr. Chem. Soc. Japan*, **23**, 115 (1949).

[17] G. T. Cori and J. Larner, *J. Biol. Chem.*, **188**, 17 (1951).

# Crystallization of Xylanase

Sir:

Sørensen[1] recently gave evidence that certain bacteria act on xylan to produce xylooligosaccharides and D-xylose. Whistler et al.[2] separated the xylan splitting enzymes of *A. foetidus* into several fractions, and studied the specific hydrolytic actions of each preparation.

The authors crystallized the xylanase which hydrolyzed xylan to xylobiose, xylotriose and higher xylooligosaccharides but produced no noticeable D-xylose, from the medium of a *Bacillus* sp. isolated from the skin of a persimmon by them.

This *Bacillus* was cultured for 7 days at 37°C in the medium containing 0.5% xylan, 0.2% fleshextract, 0.2% peptone, 0.05% $KH_2PO_4$, 0.05% $MgSO_4$ and 0.05% NaCl.

The culture medium, 5000 ml, was concentrated to 1000 ml and added 10 ml of 20% basic lead acetate. After centrifugation, ammonium sulfate was added to the supernatant solution to bring its concentration to 70% saturation. The precipitate was dissolved in 250 ml of M/15 phosphate buffer (pH 6.0), and dialyzed against distilled water containing M/1000 calcium acetate for 24 hours. Twentyfive ml of 1% rivanol was added to the dialyzed supernatant solution and the produced preipitate was discarded. The enzyme was precipitated from the supernatant solution by the addition of 500 ml of acetone and dried in vacuo. White powder, 520 mg, was obtained. This powder, 300 mg, was dissolved in 10 ml of distilled water and 3 ml of acetone was added. After removing the produced precipitate, more 2ml of acetone was added to the supernatant solution and held at 5°C. Crystals shown in Fig. 1 appeared within 1 day.

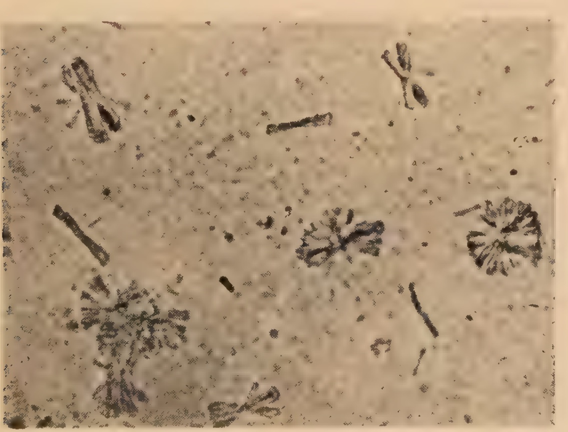

FIG. 1. Crystal of Xylanase.
× 600

TABLE I

YIELD AND ACTIVITY OF EACH FRACTION

| | Total volume | X.U. /ml | X.U. /mgN | Yield |
|---|---|---|---|---|
| Culture filtrate | 5000 ml | 25 | 36 | 100% |
| Pb-acetate treated | 1000 | 93 | 86 | 93 |
| $(NH_4)_2SO_4$ salting out | 250 | 175 | — | 35 |
| Rivanol treated | 275 | 150 | — | 33 |
| Acetone ppt. | 520 mg | | - | — |
| Crystal | 40 | — | 725 | 2.9 |

These crystals were recovered by centrifuging and drying in vacuo to yield 25 mg. The yield and the activity of each fraction were summerized in Table I.

The enzyme activity, which produced 0.135 mg of reducing sugars during 10 minutes in a reacting solution containing 1 ml of enzyme solution, 2 ml of 0.2% xylan solution and 2 ml of M/15 phosphate buffer (pH 7.0), was represented as one xylanase unit.

The activity of this crytalline xylanase was activated by the presence of M/1000 $Ca^{++}$ and $Mg^{++}$.

Paper-chromatographic analysis of the hydrolysates of corn cob xylan by this crystalline xylanase showed that xylobiose, xylotriose and higher xylooligosaccharides were the principal products and xylose was not produced.

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[1] H. Sørensen, *Physiologia Plantarum*, **5**, 183 (1952).

[2] R.L. Whistler and E. Masak, *J. Am. Chem. Soc.*, **77**, 1241 (1955).

We wish to thank Prof. Dr. K. Yamafuji for his kind guidance.

Megumu INAOKA
Hisashi SŌDA
*Department of Agricultural Chemistry,*
*Faculty of Agriculture, Ehime University*

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# An Acyl Migration in Acetohalogenoglucosamines

Sir:

In the fall of last year Micheel et al.[1] reported that the acetobromoglucosamine (3,4,6-tri-O-acetyl-2-acetylamino-2-deoxy-D-glucosyl bromide) (Ia) described in the literature is actually α-1,3,4,6-tetra-O-acetyl-D-glucosamine hydrobromide (IIa), and an interpretation that in the usual method[2] of preparation for Ia it is IIa that is produced directly but not Ia has been presented. We had been engaged in some synthetic works starting from acetobromoglucosamine and had arrived independently at the same conclusion that the so-called Ia was IIa, m.p. 210–230°, $[\alpha]_D^{13}+129°$ ($c$ 1, water), *Anal.* Calcd. for $C_{14}H_{22}O_9NBr$: C, 39.26; H, 5.18; N, 3.27. Found: C, 39.44; H, 5.41; N, 3.21, which was converted for identification into α-pentaacetyl-D-glucosamine,[3] m.p. and mixed m.p. 135°, and α-1,3,4,6-tetra-O-acetyl-2-N-(*o*-carboxybenzoyl)-D-glucosamine, m.p. 176°, *Anal.* Calcd. for $C_{22}H_{25}O_{12}N$: C, 53.33; H, 5.09; N, 2.83. Found: C, 53.05; H, 5.09; N, 2.88. Our interperation[4] of the result was that the N-acetyl group in Ia migrated after debromination at C–1 to form IIa during the preparation.

Confirmative evidence of this acyl migration was obtained with an analogue of Ia, acetochloroglucosamine (3,4,6-tri-O-acetyl-2-acetylamino-2-deoxy-D-glucosyl chloride) (Ib). Crystalline Ib[5] was relatively stable upon standing in dryness. It was converted into α-1,3,4,6-tetra-O-acetyl-D-glucosamine hydrochloride (IIb), m.p. 185°, $[\alpha]_D^{11}+140°$ ($c$ 1, water); *Anal.* Calcd. for $C_{14}H_{22}O_9NCl$: C, 43.79; H, 5.78; N, 3.65. Found: C, 44.05; H, 5.77; N, 3.41, partially upon refluxing for one hour in moist chloroform, and completely upon that in acid-containing moist chloroform. These results support our view with evidence that the N → O acetyl migration in acetohalogenoglucosamine is catalyzed by moisture and acid. After examination of reported acyl migrations in amino alcohols, we present the following mechanism for the acetyl migration in acetohalogenoglucosamine.

Starting from this reaction mechanism, we succeeded to prepare β-methyl-D-glucosaminide tetraacetate,[6] m.p. 160°, $[\alpha]_D^{18}-24°$ ($c$ 1, MeOH); *Anal.* Calcd. for $C_{15}H_{23}O_9N$: C, 49.86; H, 6.41; N, 3.89. Found: C, 49.86; H, 6.42; N, 3.88, and β-*p*-toluidine-N-D-glucosaminide tetraacetate,[7] m.p. 183°, $[\alpha]_D^{18}-2°$ ($c$ 1, MeOH), *Anal.* Calcd. for $C_{21}H_{28}O_8N_2$: C, 57.79; H, 6.46; N, 6.41; Found: C, 57.45; H, 6.45; N, 6.38, by the use of *fresh chloroform extract* of the reaction mixture[2] of pentaacetyl-D-glucosamine and HBr-acetic acid, the fact showing that Ia is formed to be active in the glycosidation by the Koenigs-Knorr reaction. Micheel et al.[1] and earlier investigators[2,5] failed to prepare glucosaminides by this reaction and two teams[6,7] of investigators who succeeded did not give detailed description on their Ia. It was noted in oursyntheses that silver oxide catalyzed the O-glucosidation from Ia, thus disproving the specific activity of mercury cyanide in this reaction as proposed by Kuhn and Kirschenlohr.[6]

---

[1] F. Micheel, F.-P. van de Kamp and H. Wulff, *Chem. Ber.*, **88**, 2011 (1955).

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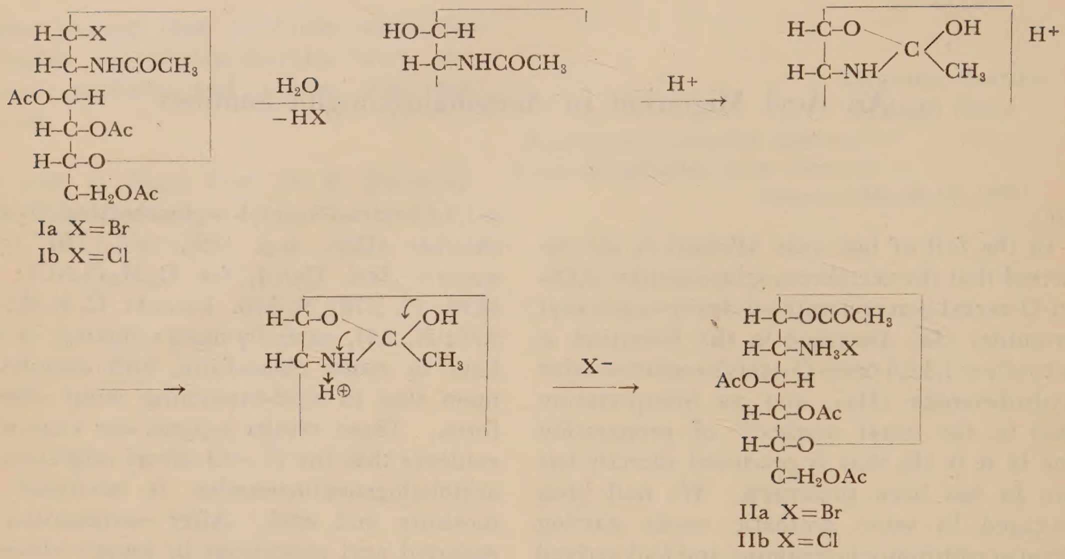

Ia X=Br
Ib X=Cl

IIa X=Br
IIb X=Cl

Attempted crystallization of the actual Ia from the above fresh chloroform extract has hitherto led to the isolation of IIa. Ia seems to undergo very rapid acyl migration in the extract.

Synthesis of some amino-disaccharides from the chloroform extract of Ia is under way, and will be published later along with the detailed description of the work outlined above.

Yoshiyuki Inouye,
Shozaburo Kitaoka,
Hideo Ochiai

*Biochemical Laboratory, College of Agriculture,*
*Kyoto University, Kyoto*

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